FINAL REPORT

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IMPROVED RISK ESTIMATES FOR CARBON TETRACHLORIDE

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I. EXECUTIVE SUMMARY

Carbon tetrachloride has been used extensively by the Department of Energy (DOE) in nuclear weapons plants, most notably at Rocky Flats, CO and Hanford, WA. High levels of carbon tetrachloride (CCl_4) at these facilities represent a potential health hazard for workers conducting cleanup operations (chiefly inhalation exposure) and for people in surrounding communities (exposure through ingestion of contaminated drinking water). The level of CCl_4 cleanup required at these sites and the associated costs are driven by current human health risk estimates that assume CCl_4 is a genotoxic carcinogen. That is, current risk estimates are based on the assumption that CCl_4 is carcinogenic because it acts directly on DNA, and therefore, no threshold dose exists below which cancer cannot be expected to occur from exposure. The overall purpose of these studies was to provide an expanded scientific database for assessing the health risk associated with human exposure to CCl_4, including data that may shed greater light on the mechanism by which CCl_4 produces liver cancer.

A three-fold approach was taken. The first approach was to evaluate the metabolism of CCl_4 by several animal species with known differences in sensitivity to the hepatotoxic and hepatocarcinogenic effects of CCl_4. Since CCl_4 must be metabolized to be toxic, comparisons of metabolic capacity may be a good predictor of species sensitivity. The metabolic capacities of rat (relatively resistant to CCl_4 hepatotoxicity), mouse and hamster (more sensitive species) were compared using: 1) *in vivo* whole-body metabolism and nose-only toxicokinetic studies; 2) *in vitro* studies with liver enzymes needed for CCl_4 metabolism (in microsomes); and 3) using microsomes prepared from the three species following repeated inhalation or drinking water exposure to CCl_4. The capacity of human liver microsomes to metabolize CCl_4 was evaluated to provide a direct comparison with the *in vitro* data obtained in the rats, mice and hamsters.

Second, the question of whether CCl_4 is a genotoxic or epigenetic carcinogen was addressed by determining whether repeated inhalation of CCl_4 concentrations known to produce liver tumors in rats and mice produced necrosis and regenerative cell growth (indicative of a non-genetic mechanisms where a threshold for toxicity and carcinogenicity is assumed to exist). The effect of repeated ingestion of drinking water containing relatively high CCl_4 concentrations (100–1000 times those allowed by law) was also evaluated. Finally, the results from the *in vivo* and *in vitro* metabolism studies were used to revise the current model for the uptake, fate and metabolism of CCl_4 and to provide an estimate for human metabolic rate constant.

This work was a collaborative effort between the Lovelace Respiratory Research Institute (LRRI) and Pacific Northwest National Laboratory (PNNL). The *in vitro* metabolism, whole-body metabolism, and modeling efforts were performed at PNNL. The inhalation and drinking water exposures, nose-only toxicokinetic studies, and evaluations of hepatic histopathology and regenerative cell proliferation were conducted at LRRI.

The major results of our collaborative studies are:

- No previous studies have comprehensively assessed the rates of CCl_4 metabolism in rodent species other than the rat. The combined results of our *in vitro* and *in vivo* metabolism and toxicokinetic studies indicate that the relative abilities of the species investigated to metabolize CCl_4 is hamster > mouse > rat ≈ man. Since rats are the rodent species least sensitive to the toxic effects of inhaled CCl_4, and metabolism is required for CCl_4 to cause toxic effects in the liver, these data strongly suggest that humans may also be relatively resistant to CCl_4 hepatotoxicity.
- The relative sensitivities of the rodent species to the effects of repeatedly inhaled CCl_4 is mouse > hamster > rat. These data, in conjunction with the data on relative metabolic capacity of the three species suggest that the ability to metabolize CCl_4 to reactive intermediates is not the only factor mediating sensitivity or resistance to CCl_4 exposure. Existing levels of antioxidant capacity and/or the ability of CCl_4 exposure to increase levels of antioxidants in the body are also likely to play a role in determining relative species sensitivity. The effect of CCl_4 exposure on antioxidant capacity in the rodents was not investigated in these studies.
- Results of the *in vitro* species comparison studies of CCl_4 metabolism suggest that cytochrome P450 isozyme 2E1 is the major human enzyme responsible for CCl_4 metabolism at low, environmentally relevant concentrations. At higher CCl_4 exposure levels, isozyme 3A and possibly other cytochrome P450 isozymes may contribute to CCl_4 metabolism.
- The *in vivo* metabolic constants determined in the whole-body metabolism studies, coupled with the metabolic rate constants determined using rats, mice, hamsters and human liver preparations *in vitro,* have been used to estimate *in vivo* metabolic rates for humans. The estimated maximum velocity rate of CCl_4 metabolism in humans is 1.49 mg/hr/kg body weight. The affinity constant is estimated at 0.25 mg/L.

- Repeated ingestion of drinking water containing CCl_4 concentrations 100 times greater than permitted by law (5 ppb) resulted in no hepatotoxic effects or regenerative cellular proliferation in rats, mice and hamsters. Repeated ingestion of water containing CCl_4 at concentrations 1000 times higher than permitted by law resulted in no hepatotoxicity in rats or mice and only transient early effects in hamsters. Only rats ingesting 500 and 5000 ppm CCl_4 showed small, transient increases (<25%) in the level of enzyme activity specifically associated with CCl_4 metabolism. Therefore, the toxic and metabolic effects of ingestion of relatively high levels of CCl_4 in drinking water were minimal.
- Hepatocellular proliferation did not occur in the absence of hepatocellular necrosis. Hepatocellular necrosis and regenerative cell proliferation occurred in mice at 20 and 100 ppm CCl_4, concentrations resulting in significantly increased numbers of hepatocellular adenomas and carcinomas in mice in a 2-year inhalation bioassay. Hepatocellular necrosis and a small, but not significant, increase in cell proliferation occurred in rats inhaling 100 ppm CCl_4, a concentration resulting in significantly increased numbers of hepatocellular adenomas and carcinomas in rats in a 2-year inhalation bioassay. The amount of necrosis in rats inhaling 100 ppm CCl_4 was greater at 12 weeks than at earlier times, suggesting that with continued exposure necrosis and regenerative cell proliferation may have increased. Overall, these data support the hypothesis that CCl_4 induces tumors only at concentrations resulting in cell injury, death, and regenerative proliferation.
- The no adverse effect level for inhaled CCl_4 in our studies is estimated at 5 ppm, the current Threshold Limit Value established by the American Conference of Government Industrial Hygienists (ACGIH). The no adverse effect level in our studies for drinking water can be safely estimated at 500 ppb, a value 100 times higher than the 5 ppb limit established by the Environmental Protection Agency (EPA).

II. RESEARCH OBJECTIVES

Carbon tetrachloride has been used extensively within the DOE nuclear weapons facilities. Rocky Flats was formerly the largest volume consumer of CCl_4 in the United States using 5000 gallons in 1977 alone (Ripple, 1992). At the Hanford site, several hundred thousand gallons of CCl_4 were discharged between 1955 and 1973 into underground cribs for storage. Levels of CCl_4 in groundwater at highly contaminated sites at the Hanford facility have exceeded

the drinking water standard of 5 ppb by several orders of magnitude (Illman, 1993). High levels of CCl_4 at these facilities represent a potential health hazard for workers conducting cleanup operations and for surrounding communities. The level of CCl_4 cleanup required at these sites and associated costs are driven by current human health risk estimates, which assume that CCl_4 is a genotoxic carcinogen. The overall purpose of these studies was to improve the scientific basis for assessing the health risk associated with human exposure to CCl_4.

Specific research objectives of this project were to: 1) compare the rates of CCl_4 metabolism by rats, mice and hamsters *in vivo* and extrapolate those rates to man based on parallel studies on the metabolism of CCl_4 by rat, mouse, hamster and human hepatic microsomes *in vitro*; 2) using hepatic microsome preparations, determine the role of specific cytochrome P450 isoforms in CCl_4-mediated toxicity and the effects of repeated inhalation and ingestion of CCl_4 on these isoforms; and 3) evaluate the toxicokinetics of inhaled CCl_4 in rats, mice and hamsters. This information has been used to improve the physiologically based pharmacokinetic (PBPK) model for CCl_4 originally developed by Paustenbach *et al.* (1988) and more recently revised by Thrall and Kenny (1996). Another major objective of the project was to provide scientific evidence that CCl_4, like chloroform, is a hepatocarcinogen only when exposure results in cell damage, cell killing and regenerative proliferation. In combination, the studies were intended to provide the exact types of information needed to enable refined cancer risk estimates for CCl_4 under the new EPA guidelines for risk assessment.

III. ALTERATIONS IN CYTOCHROMES P450 2E1 AND 2B EXPRESSION IN RESPONSE TO LOW-DOSE CARBON TETRACHLORIDE EXPOSURE BY INHALATION OR DRINKING WATER

A. Background

Carbon tetrachloride is reduced by cytochromes P450 (CYP450) to form a highly toxic trichloromethyl radical. This radical can dismutate to form chloroform ($CHCl_3$) or can react with dioxygen and/or cellular macromolecules to produce lipid peroxidation and disrupt cellular processes (McGregor and Lang, 1996). At high doses, CCl_4 induces acute liver damage and increases the risk of hepatocarcinogenesis (McGregor and Lang, 1996). CCl_4 acts as a mechanism-based inhibitor of the CYP450 forms that catalyze its metabolism and at high doses clearly suppresses CYP450 levels (Chadwick *et al.*, 1988). In addition, lipid peroxidation resulting from high-dose CCl_4 exposure may further inactivate CYP450 (de Groot and Noll,

1989). In contrast to effects observed at high doses, Chadwick *et al.* (1988) reported that a single low-dose administration of CCl_4 modestly increases CYP450-associated activities in rats. Since induction of bioactivating CYP450 forms is associated with increased susceptibility to CCl_4 toxicity (Raucy *et al.*, 1993), changes in CYP450 levels could be an important factor in determining toxic responses after extended exposure to low-doses of CCl_4. Therefore, it is important to determine if extended exposure to low doses of CCl_4 may be efficacious in inducing CYP450 responses since such increases may increase toxic responses in chronically exposed animals. Similarly, in order to determine if such effects are likely to relate to humans, it is necessary to determine if species other than rats are also responsive.

CYP2E1 is likely to be the primary enzyme responsible for bioactivating CCl_4 in most rodents (Raucy *et al.*, 1993; Wong *et al.*, 1998), although CYP2B forms may also be important in some circumstances (Salmon *et al.*, 1985; Frank *et al.*, 1982). Therefore, any effects of low-dose CCl_4 exposure on these CYP450s may alter CCl_4 metabolism and toxicity following repeated exposure. Therefore, we determined the effects on CYP2E1 and CYP2B protein levels and associated catalytic activities in rats, mice and hamsters following repeated exposure to CCl_4 by inhalation or drinking water. We find that although low doses of CCl_4 can induce these CYP450 proteins, these changes occur in the absence of large changes in catalytic activity.

B. Methods

1. Experimental Design

Groups of 10 male F344/Crl rats, $B6C3F_1$ mice and Syrian hamsters were exposed by inhalation to 0, 5, 20 or 100 ppm CCl_4 6 hours/day, 5 days per week for 1 or 12 weeks. On the day following the end of exposure, groups of five animals/species/CCl_4 exposure level were sacrificed by intraperitoneal injection of an overdose of pentobarbital. Livers were removed, weighed and immediately placed in buffer. Hepatic microsomes were prepared as described by Zangar *et al.* (1996), aliquoted and frozen immediately in liquid nitrogen. For rats and hamsters, 5 g of liver was used for microsomal preparation. For mice, the whole liver was used. Microsome samples were stored at –80°C until used.

To determine the effects of repeated ingestion of CCl_4-contaminated drinking water on hepatic CYP450, groups of 10 male F344/Crl rats, $B6C3F_1$ mice and Syrian

hamsters were administered 0.5 or 5 ppm CCl_4 in drinking water for 1 or 4 weeks. Control animals were administered pure water under the same conditions. After 1 or 4 weeks of exposure, groups of five animals/species/CCl_4-drinking-water concentration were sacrificed and microsomes prepared as described above.

2. Immunoblot Analyses

Microsome protein concentrations were determined as described (Bradford, 1976). The Western gels and transfer to nitrocellulose were undertaken as described (Zangar *et al.*, 1993). Preparation of the CYP2B antibody was described previously (Zangar *et al.*, 1993). The CYP2E1 antibody was purchased from Gentest (Woburn, MA). Secondary antibodies that were alkaline phosphatase conjugated were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Protein bands were imaged and quantified using the AttoPhos substrate (JBL Scientific, San Luis Obispo, CA) and a Vistra FluorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

3. Catalytic Assays

Ethoxytrifluorocoumarin and benzyloxyresorufin O-deethylases (EFCOD and BzROD, respectively) were undertaken using 5 μM substrate, 50 μg of microsomal protein, 100 mM sodium phosphate buffer, pH 7.4, 0.4 mg bovine serum albumin/mL, 10 μM α-naphthoflavone and 1 mM NADPH, in a final volume of 250 μL. The α-naphthoflavone was used to inhibit CYP1A2, which could have interfered with the assessment of CYP2B catalytic activity by these assays (Burke *et al.*, 1985; Ekins *et al.*, 1997). In agreement with this, preliminary studies indicated that α-naphthoflavone inhibited 30 to 60% of these CYP2B-associated activities in hepatic microsomes from untreated rats but was without significant effect on samples from phenobarbital-treated rats (data not shown), which have highly induced levels of CYP2B (Waxman and Azaroff, 1992). Samples were prewarmed to 37°C for 3 minutes prior to initiating the reaction by addition of NADPH. For samples used to determine background fluorescence, an equal volume of water without NADPH was added. Samples were then incubated at 37°C for 20 minutes, at which time 50 μL of 20% trichloroacetic acid was added to terminate the reaction. The acid-precipitated protein was pelleted by centrifugation and the supernatant transferred to a tube containing 1.2 mL of 200 mM Tris base (no pH adjustment).

For the EFCOD samples, fluorescence was determined using an excitation of 385 nm and emission of 502 nm or, for the BzROD, excitation of 530 nm and emission of 585 nm. Following the initial fluorescence reading, 100 pmole of trifluormethylumbelliferone (EFCOD samples) or 50 pmole of resorufin (BzROD samples) was spiked into the sample, and the fluorescence was determined again. The change in fluorescence was used to determine quenching and the ratio between moles of product and the fluorescence reading.

Chlorzoxazone was dissolved directly in sodium phosphate buffer without the use of solvents. Since glycerol inhibits CYP2E1 activity, microsomes initially stored in glycerol-containing buffer were centrifuged at 100,000 *g* and resuspended in glycerol-free sodium phosphate buffer on the day of the assay. Chlorzoxazone 6-hydroxylase activity was determined using 200 µM chlorzoxazone, 200 µg of microsomal protein, 100 mM sodium phosphate buffer, pH 7.4 and 1 mM NADPH in a final volume of 200 µL. Samples were prewarmed to 37°C for 3 minutes, at which time the reaction was initiated by addition of NADPH. Samples were then incubated at 37°C for 20 minutes, at which time 200 µL of 4°C acetonitrile:methanol:HCl (90:10:0.2; v:v:v) was added to terminate the reaction. The precipitated protein was pelleted by centrifugation and the supernatant analyzed using a Waters' (Milford, MA) HPLC system that incorporated a 600E gradient controller and a dual-pump system, a Waters 996 photodiode array detector and a Zorbax 3 × 150 mm, XDB-C18 column (MAC MOD Analytical, Chadds Ford, PA). Absorbance of the 6-hydroxychlorzoxazone was determined at 595 nm. A linear gradient starting at 10% acetonitrile/90% water, increasing to 80% acetonitrile over 10 minutes and then maintained for an additional 2 minutes was used.

C. Results

1. Microsomal Protein Content

Increases in hepatic microsomal protein content were observed following treatment with certain CYP450 inducers such as phenobarbital, while decreased microsomal protein content is typically associated with hepatic toxicity. In rats, microsomal protein levels were increased 45% and 63% following 5 day inhalation exposure to 20 and 100 ppm CCl_4, respectively, but were not significantly altered in the 5-day, 5 ppm inhalation group, the 12-week inhalation groups or drinking water exposure groups (Fig. 1). In hamsters, 100 ppm CCl_4 inhalation exposure decreased microsomal protein levels by 33% and 54% in the 5-day and 12-

week exposure groups, respectively. In contrast to the inhalation exposure effects, in hamsters exposed to 0.5 and 5 ppm CCl_4 for 1 week in the drinking water, microsomal protein levels were increased 44% and 76%, respectively, although 4-week drinking water exposure was without significant effect. Mice did not show any significant change in microsomal protein content under any CCl_4 exposure condition examined here. These data demonstrate that microsomal protein was only induced after the shorter, 1-week exposures but that longer exposures of 4 weeks in the drinking water or 12 weeks by inhalation did not significantly increase this parameter. Microsomal protein levels decreased in hamsters in the 100 ppm inhalation treatment, the only dose level that produced increases in toxic indices (i.e., hepatocellular necrosis, serum alanine amino transferase levels, and percent 5-bromo-2′-deoxyuridine [BrdU; a thymidine analog] positive cells; Nikula *et al.*, 1998). Although mice also exhibited hepatotoxicity at the 20 and 100 ppm CCl_4 inhalation doses, the levels of necrosis were less than in hamsters exposed to 100 ppm CCl_4 (Nikula *et al.*, 1998), and no differences in microsomal protein levels were observed.

2. CYP2E1 Effects

Western blot analyses indicated that CYP2E1 protein levels were weakly induced (up to 2-fold) in all three species at subtoxic inhalation exposure levels (i.e., those that did not increase hepatocellular necrosis, serum alanine amino transferase levels and percent BrdU positive cells; Nikula *et al.*, 1998) by both administration routes (Figs. 2 and 3). However, not all exposure regimens induced this protein, indicating that exposure route and duration may interact to determine levels of CYP2E1 induction. At clearly toxic CCl_4 levels (inhalation exposure levels of 20 and 100 ppm in the mice or 100 ppm in the hamster), CYP2E1 protein levels were generally decreased relative to untreated animals. An exception to this observation was the ~50% increase in CYP2E1 levels observed in mice exposed by inhalation to 20 ppm CCl_4, a dose that produced mild toxicity in this species (Nikula *et al.*, 1998). Another exception was that CYP2E1 levels were not decreased in mice exposed by inhalation to 100 ppm CCl_4 relative to control mice, although these levels were lower than the 5 and 20 ppm exposure groups, which exhibited increased CYP2E1 protein levels. These results suggest that the CCl_4-mediated induction of CYP2E1 observed at lower doses may be partially or completely offset by CYP2E1 suppression at higher exposure levels.

Treatments that reduced CYP2E1 protein levels in mice and hamsters also exhibited reduced chlorzoxazone 6-hydroxylase (CHZ OHase), a CYP2E1-associated activity (Figs. 2 and 3). The relationship between CHZ OHase activity and immunodetectable protein levels was more variable, however, in treatments in which CYP2E1 protein levels were elevated. CHZ OHase activity was significantly increased 16% and 23% in rats exposed to 0.5 and 5 ppm CCl_4 in the drinking water, respectively, in agreement with increases in immunodetectable CYP2E1 in these rats. In other cases, increases in immunodetectable CYP2E1 protein levels were not accompanied by increased CHZ OHase activity. Twelve-week inhalation exposures of 100 ppm actually resulted in decreased CHZ OHase activity in rats and mice in spite of Western blot data that indicated that CYP2E1 protein levels were unchanged or increased in these treatments. The most likely explanation for the discrepancies between CYP2E1 protein levels and activity is that some of the CYP2E1 detected in the immunoblots had been inactivated by CCl_4 metabolites in these treatments.

3. CYP2B Effects

Results for CYP2B were similar to those obtained for CYP2E1. CYP2B was induced as much as 3-fold by CCl_4 exposure in all three species, although only certain treatment regimens were effective in inducing this CYP450 subfamily (Figs. 4 and 5). In mice and hamsters, higher inhaled concentrations of CCl_4 (e.g., doses that produced clear toxicity) typically resulted in sharply reduced CYP2B protein levels. An exception to this was observed in mice exposed for 5 days to 20 ppm CCl_4, a treatment regimen that increased CYP2B protein levels.

It is difficult to select a single substrate for assessing CYP2B activity across species (see Nims and Lubet, 1996, for review). Therefore, we used two enzymatic assays, EFCOD and BzROD, to assess CYP2B activity in this study. These two assays closely paralleled each other in the rat. They also were typically in agreement in mice, although the BzROD activity generally paralleled the immunodetectable levels of CYP2B protein more closely in mice. The BzROD activity varied remarkably (over 10-fold) between groups of hamsters from different experiments (compare the hamster BzROD data in Figs. 4 and 5) and did not correlate well with the EFCOD activity in this species. This range of BzROD activity in hamsters was reproducible and not an artifact of analyses performed on different days. Decreases in immunodetectable CYP2B protein levels were consistently associated with

decreases in one or both catalytic activities in all species. In rats, the CYP2B-associated activities were decreased approximately 60% and 30% following 5-day and 12-week inhalation exposures, respectively. This decrease in activity occurred in spite of immunoblot evidence that the CYP2B protein levels were increased approximately 60% and 200%, respectively, in these same treatments. Similarly, other increases in CYP2B protein levels detected in Western blots were not associated with parallel increases in these catalytic activity. These data further support the concept that induction of CYP450 apoprotein by CCl_4 can be at least partially offset by catalytic inactivation.

D. Discussion

In this study, we examined the effects of repeated CCl_4 exposure on CYP450 expression in rats, mice and hamsters. At CCl_4 doses that were clearly associated with hepatotoxicity, CCl_4 typically suppressed CYP2E1, CYP2B and microsomal protein content. In contrast, some of the lower dose CCl_4 treatment regimens induced CYP450 protein levels in each species (data summarized in Table 1). This result clearly establishes that CCl_4 functions as a CYP450 inducer at low doses. Even so, the level of induction observed after CCl_4 exposure was modest compared to that observed with many other xenobiotic CYP450 inducers. Furthermore, the increases in immunodetectable protein levels were not associated with large or significant increases in catalytic activities associated with these CYP450s. Indeed, in rats exposed by inhalation to 100 ppm CCl_4, increases in CYP2E1 or CYP2B protein levels were accompanied by decreases in the associated enzymatic activities (Table 1). This result suggests that CYP450 inactivation resulting from CCl_4 metabolism accounts for the discrepancies between activity and protein levels. Human CYP450s appear to be less susceptible than those of rats to inactivation by CCl_4, such that approximately 200 CCl_4 molecules must be metabolized per inactivated CYP450 enzyme in humans, but only 26 CCl_4 molecules must be metabolized in rats (Manno *et al.*, 1988, 1992). Therefore, CYP450 inactivation by CCl_4 in humans may be less significant than in rats, and induction of catalytic activity could be correspondingly greater.

It is interesting to note that the rat, which was less susceptible to CCl_4 toxicity (Section VII, this report), typically exhibited the greatest induction response, as demonstrated by increases in immunodetectable CYP2B and CYP2E1 protein levels, catalytic activity or microsomal protein content. Of the rodent species, the rat is the least efficient at metabolizing CCl_4, as demonstrated with microsomal studies and whole-body uptake studies (see Sections IV

and VI, this report). Since CCl_4 toxicity is associated with metabolic conversion to the toxic trichloromethane radical, these data suggest that the rat's ability to tolerate higher doses of CCl_4 is the result of poor metabolic capacity. Likewise, the greater inductive effects observed in the rat may reflect an ability to tolerate higher CCl_4 concentrations without the development of oxidative stress. Based on studies employing hepatic microsomes, humans are inefficient at metabolizing CCl_4, similar to rats (Section IV), suggesting that humans may exhibit a CCl_4 induction response similar to that observed in the rat and still be resistant to CCl_4-induced hepatotoxicity.

An alternative possibility is that the CYP450 induction response we measured involves induction of a broader spectrum of enzymes than the cytochrome P450s alone. It is possible that enzymes contributing to antioxidant responses were also induced; therefore, this broad inductive response may be important in preventing toxicity in some species. Support for this hypothesis is demonstrated by the increase in total microsomal protein in rats exposed by inhalation to 100 ppm CCl_4 for 5 days, an increase that cannot be accounted for by the increases in CYP2E1 and CYP2B proteins alone. Therefore, the rat might exhibit less toxicity as a result of induction of antioxidant proteins. In fact, species differences in susceptibility to CCl_4-induced hepatotoxicity, with mice exhibiting toxicity at a lower concentration (20 ppm) than hamsters or rats (Nikula *et al.*, 1998), are in contrast to the species-specific differences in microsomal protein induction. This concept is supported by an analysis of CCl_4 metabolic constants in hamsters, which exhibit a higher capacity (V_{max}) than either rats or mice and an affinity (K_m) that is similar to mice but lower than the rat (Section VI, this report). Since hamsters are more efficient at metabolizing CCl_4, this species would be expected to be at least as susceptible as mice to CCl_4 toxicity. Consistent with this concept, following a 1-week, 100 ppm inhalation exposure, hamsters exhibit markedly greater hepatotoxicity than do mice (Nikula *et al.*, 1998). However, after exposures to 100 ppm for 4 or 12 weeks, the hamster's toxic response decreases while mice respond similarly to that observed at 1 week. In addition, at exposures of 20 ppm for 1, 4 or 12 weeks, mice, but not hamsters, exhibited hepatotoxicity. This suggests that hamsters either have a better basal antioxidant system than the mouse or that a protective system is rapidly induced in response to CCl_4 exposure. However, when this antioxidant system is depleted by higher doses of CCl_4, the greater metabolic capacity of the hamster results in increased radical formation and greater toxicity than observed in the mouse.

Following high-dose CCl_4 exposure, inactivated CYP2E1 is rapidly degraded by the ubiquitin-proteasome pathway, such that 75% of the immunodetectable CYP2E1 protein levels are lost 1 hour after high-dose CCl_4 treatment (Tierney *et al.*, 1992; Roberts, 1997). Consistent with this model, we observe decreased levels of CYP2E1 protein at doses of CCl_4 that are clearly toxic. However, at lower CCl_4 doses, decreases in chlorzoxazone hydroxylase activity in treatments where immunodetectable levels of CYP2E1 were unchanged or even increased, suggest that the microsomal CYP2E1 pool may be partially inactivated without an accelerated loss of apoprotein. These data suggest that accelerated degradation of inactivated CYP2E1 protein may be partially dependent on the oxidative damage that develops following high-dose exposure to CCl_4. Lipid peroxidation resulting from CCl_4 metabolism provides an additional mechanism by which CYP450 is inactivated (de Groot and Noll, 1989) and may also be important in accelerated CYP2E1 protein degradation observed after high-dose CCl_4 exposure.

Overall, these studies show that CCl_4 is a weak CYP2E1 and CYP2B inducer at lower concentrations, but suppresses their activity at toxic concentrations. The low-dose CCl_4-meditated CYP450 induction, however, is unlikely to have a large effect on CCl_4 bioactivation in rodents because protein induction appears to be largely offset by mechanism-based inactivation of the induced CYP450s.

IV. CYTOCHROME P450 2E1 IS THE PRIMARY ENZYME RESPONSIBLE FOR LOW-DOSE CARBON TETRACHLORIDE METABOLISM IN HUMAN LIVER MICROSOMES

A. Background

Although CYP2E1 is suspected of being the major catalyst of CCl_4 reduction in humans (Guengerich *et al.*, 1991), the role of the various human CYP450 forms in CCl_4 metabolism over a range of CCl_4 concentrations has not been established. An understanding of which CYP450 forms contribute to CCl_4 metabolism would provide insight to the molecular factors that influence individual susceptibility to CCl_4 toxicity, as well as how environmental influences might alter susceptibility. In this study, we examined the contribution of human CYP450 forms to CCl_4 metabolism using expressed enzymes and inhibitory antibodies and chemicals. In addition, we compared rates of CCl_4 metabolism in hepatic microsomes obtained

from human and rodent species in order to better understand how such differences relate to susceptibility to CCl_4 toxicity.

B. Methods

1. Preparation of Microsomes

Hepatic microsomes from rats, mice and hamsters were prepared as described (Zangar *et al.,* 1996), aliquoted and frozen immediately in liquid nitrogen. Since glycerol in the storage buffer may inhibit CYP2E1 activity, rodent microsomes were pelleted by centrifugation, then suspended in glycerol-free sodium phosphate buffer shortly before assaying for catalytic activity. Human microsomes were prepared in sucrose buffer as described (Kim *et al.*, 1996) from three liver sections obtained from IIAM (Exton, PA). These liver sections had been perfused with University of Wisconsin medium and contained viable hepatocytes. The liver designated HL1 was from a 109 kg, 6'0", black male, age 25 who died of a gunshot wound. HL2 was from a 90 kg, 5'10" Caucasian male, age 69 who died of cardiopulmonary arrest. HL3 was from a 73 kg, 5'4" Caucasian female, age 68, who died of a brain stem infarction. Protein concentrations were determined as described (Bradford, 1976).

2. Determination of CCl_4 Metabolic Rate Constants in Liver Microsomes and with Expressed CYP450s

CCl_4 metabolism was determined under anaerobic conditions by determination of $CHCl_3$ formation similar to that described (Andersen *et al.*, 1996). Since CYP2E1 is inhibited by many solvents, a saturated solution of CCl_4 was prepared in degassed 0.1 M sodium phosphate buffer, pH 7.4 without the use of additional solvents. The precise concentration of this solution was determined daily for each experiment by preparing three replicate 1:1000 dilutions of the CCl_4 stock in methanol. Then 1 µL of these diluted samples or of 0, 0.078, 0.31, 1.25, 5 and 20 µM CCl_4 standards were analyzed by gas chromatography (GC) and the concentration of the aqueous stock calculated. CCl_4 and $CHCl_3$ levels were analyzed using a Hewlett Packard (Wilmington, DE) 5890 Series II GC with a 15 m, 0.32 mm i.d. DB5 column and an electron capture detector. Inlet, column and detector temperatures were 170°C, 40°C and 300°C, respectively. Once the concentration of the stock solution was determined, the CCl_4-saturated solution was diluted to the desired concentration(s) in degassed 0.1 M sodium

phosphate buffer, pH 7.4. These diluted solutions were prepared and stored in argon-purged, sealed vials that were filled to the top to prevent loss of CCl_4 or uptake of oxygen.

Incubation conditions were as follows: 1 mg microsomal protein/mL, 0.1 M sodium phosphate buffer, pH 7.4, 1 mM NADPH, 60 mM glucose, 10 units glucose oxidase/mL, 2000 units catalase/mL and various concentrations of CCl_4 in a final volume of 0.5 mL. The glucose, glucose oxidase and catalase served as an oxygen scavenger system (Andersen *et al.*, 1996). All reagents except the CCl_4 were mixed and sealed in 1.8 mL, crimp-cap GC vials and stored on ice. Samples were degassed by blowing argon through the vials for 10 minutes using 27 g, 0.5" needles for both the gas inlet and vent. Vials were pre-incubated for 5 minutes in a 37°C water bath with shaking at 150 rpm. Vials were vented and the CCl_4 solution added using a Hamilton syringe. Typically, 50 µL of CCl_4 solution was added. Vials were then placed back in the shaking incubator for another 5 minutes. The reaction was terminated by the addition of 70 µL of 50% H_2SO_4. Three or four replicate samples were analyzed for each data point. Samples were then placed back in the shaking water bath for an additional hour, at which time 5 µL of headspace gas was analyzed using the GC procedure described above. In order to determine the molar amount of $CHCl_3$ produced, a standard curve was prepared using vials in which 0, 0.25, 0.5, 1, 2, 4 or 8 nmole of $CHCl_3$ in 10 µL methanol was added. These vials were prepared in a manner identical to the vials used for the CCl_4 metabolic analysis except that the 70 µL of H_2SO_4 was added immediately prior to the start of 37°C incubation, and one $CHCl_3$ solution was added after the 5 minute pre-incubation instead of a CCl_4 solution.

3. <u>$CHCl_3$ Microsomal Metabolism</u>

Microsomal $CHCl_3$ metabolism was determined under identical incubation conditions as used for the microsomal CCl_4 metabolic studies. Ten µL of stock $CHCl_3$ solution in sodium phosphate buffer was added to each vial after the 5 minute pre-incubation, such that the assay concentration was 10 µM $CHCl_3$.

4. <u>Studies with Inhibitory Antibodies or Chemicals</u>

Studies with inhibitory antibodies were conducted in each of the three human liver microsome samples. Undiluted microsome stocks were incubated on ice with 3 µL of 25 mM Tris, pH 7.5 or inhibitory antibody per 100 µg of microsomal protein for 15 minutes.

Studies with the chemical inhibitors were undertaken using human liver HL2. Chemical inhibitors were added to the sealed and degassed vials using a 10 µL Hamilton syringe prior to the start of incubation. Chemical inhibitors were dissolved in acetonitrile such that assay concentrations were 10 µM inhibitor and 0.1% acetonitrile. Incubation conditions in the inhibitor studies were identical to those described above, except that 0.5 mg microsomal protein/mL was used. Three or four replicate samples were analyzed for each data point. The relative amount of inhibition was determined by comparing $CHCl_3$ formation in samples containing antibodies or chemical inhibitors to $CHCl_3$ levels in identical samples without inhibitor.

C. Results

1. Metabolism of CCl_4 by Human, Rat, Mouse and Hamster Microsomes

Initial studies of CCl_4 metabolism were undertaken using microsomes from humans, rats, mice or hamsters. Microsomes from three individuals were pooled for each species and a range of CCl_4 concentrations were examined. Data from these studies are presented in Figure 6 and Table 2. These studies indicated that human liver microsomes had a V_{max} and K_m of 2.26 nmole/minute/mg and 56.8 µM, respectively. Overall, the differences in K_m and V_{max} did not vary more than ~2-fold between species, although both the mouse and hamster had slightly lower K_m values than the human or rat, while hamsters had the highest V_{max} of any species examined. Therefore, humans may be less efficient at metabolizing CCl_4 than the three rodent species examined here, but these differences are not dramatic.

2. Lack of Anaerobic $CHCl_3$ Metabolism

The analysis of CCl_4 microsomal metabolites did not detect any hexachloroethane, suggesting that levels of trichloromethane radicals formed in our incubation system were too low for significant amounts of radical recombination to occur. Still, it is possible that subsequent metabolism of the CCl_4 metabolite, $CHCl_3$, could potentially skew results by decreasing the apparent rate of CCl_4 metabolism. Testai *et al.* (1996) showed that anaerobic metabolism of $CHCl_3$ was not observable at 100 µM $CHCl_3$, although some metabolism could be observed under anaerobic conditions at 5000 µM $CHCl_3$. To determine if there was subsequent metabolism of $CHCl_3$ under conditions directly relevant to our incubation

conditions, studies were undertaken using 10 μM $CHCl_3$, a concentration of metabolite achieved in the human microsome incubations when CCl_4 concentrations were saturating. There was no detectable loss (less than 3% change in all species) of $CHCl_3$ or detectable levels of CH_2Cl_2 in incubated microsomes from humans, rats, mice or hamsters (data not shown). Similarly, there was no detectable CH_2Cl_2 formed in any study presented below, suggesting that $CHCl_3$ metabolism was not significant. These results indicate that secondary metabolism of $CHCl_3$ was not a confounding factor in these studies.

3. Metabolism of CCl_4 by Expressed Human CYP2E1 and CYP2B6

The ability of human CYP2E1 and CYP2B6 to reduce CCl_4 was determined using heterologously expressed enzymes. Expressed CYP2E1 reduced CCl_4 under anaerobic conditions with a V_{max} of 8.96 nmole $CHCl_3$/minute/nmole CYP2E1 and a K_m of 1.91 μM (values were derived from the graph in Fig. 7, using SigmaPlot software). Expressed CYP2B metabolized CCl_4 with a V_{max} of 2.25 nmole $CHCl_3$/minute/nmole CYP2B6 and a K_m of 38.3 μM (graph not shown). These data indicate that CYP2E1 has both greater affinity and capacity to metabolize CCl_4 than does CYP2B6.

4. Inhibition of CCl_4 Metabolism by CYP2E1 and CYP2B6 Antibodies

Antibody inhibition studies were undertaken using two CCl_4 concentrations, 17 μM and 530 μM, and three human microsome preparations. At 17 μM CCl_4, CYP2E1 antibodies inhibited from 64% to 83% of the total CCl_4 metabolism, while at 530 μM CCl_4, 36% to 75% inhibition was observed (Fig. 8). The CYP2E1 antibody used can be expected to inhibit a maximum of approximately 80% of total CYP2E1 activity, but does not inhibit the activity of nine other human CYP450s (Gelboin *et al.*, 1996). Therefore, the CYP2E1 antibody inhibition data suggest that at 17 μM CCl_4 or less, CYP2E1 is the primary catalyst of CCl_4 metabolism in the three human livers examined here. At 530 μM CCl_4 or higher, CYP2E1 contributed significantly to CCl_4 metabolism in microsomes from all three livers, but it appears that other CYP450 forms are also likely to be important.

An inhibitory monoclonal CYP2B6 antibody did not significantly affect CCl_4 metabolism in any human microsome sample at either 17 or 530 μM CCl_4 (data not shown). This result is consistent with our data indicating that expressed CYP2B6 is an

inefficient catalyst of CCl_4 reduction (see above) and reports that CYP2B6 expression is generally low in human liver (Guengerich, 1995).

5. Inhibition of CCl_4 Metabolism by Chemical Inhibitors

In order to examine for microsomal CYP450s other than CYP2E1 that may contribute to CCl_4 metabolism at high substrate concentrations, further studies were undertaken using chemical inhibitors that have been reported to be selective for certain CYP450 forms or subfamilies. These studies were undertaken in microsomes obtained from the human liver HL2 since these microsomes exhibited the least amount of inhibition in the presence of CYP2E1 antibodies at 530 μM CCl_4 (Fig. 8). Acetonitrile (0.1%) was used as a carrier solvent for the chemical inhibitors. In agreement with a report that acetonitrile concentrations of 0.2% or higher did not inhibit human microsomal enzymatic activities associated with CYP1A2, CYP2C6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 (Chauret *et al.*, 1998), we found that this solvent did not inhibit CCl_4 metabolism in this study (Fig. 9). No inhibition was observed with α-naphthoflavone or sulfaphenazole, inhibitors of CYP1A and CYP2C9, respectively. Clotrimazole did inhibit CCl_4 metabolism 23%, suggesting that CYP3A may contribute to CCl_4 metabolism at high CCl_4 concentrations in some individuals.

D. Discussion

In rodent species, CYP2E1 and CYP2B have been implicated in CCl_4 metabolism. In this study we found that CYP2E1 is a high-affinity catalyst responsible for low-dose CCl_4 metabolism. Studies employing an inhibitory CYP2E1 antibody confirmed that the majority of metabolism at 17 μM CCl_4 in human microsomes was dependent on CYP2E1. In order to relate this CCl_4 concentration with actual exposure conditions, a PBPK model was used (Paustenbach *et al.*, 1988; Thrall *et al.*, 1996). This analysis indicated that a maximal liver concentration of 17 μM CCl_4 would be achieved on a daily basis in a 200 g rat following a single or repeated 8-hour inhalation exposure to approximately 37 ppm CCl_4 (Dr. Karla Thrall, personal communication). This level of exposure is not hepatotoxic in rats, although it would be toxic in a more susceptible species such as the mouse (Section VII, this report). Therefore, it seems likely that human metabolism of CCl_4 at doses that are not hepatotoxic is primarily catalyzed by CYP2E1.

The importance of CYP2E1 in CCl_4 metabolism in humans provides insight into the molecular basis for the association between alcohol consumption and increased susceptibility to CCl_4 toxicity observed in previous studies. In one such study, Tomenson *et al.* (1995) examined individuals exposed to occupational levels of CCl_4 and demonstrated that alcohol use was associated with alterations in several biochemical and hematological indices of hepatotoxicity. Manno *et al.* (1996) also found that chronic ethanol consumption predisposed humans to CCl_4 toxicity. Since ethanol is known to induce CYP2E1 (Koop and Tierney, 1990; Zangar *et al.*, 1995), it is likely that the increased response to CCl_4 exposure in individuals who consume ethanol is associated with enhanced CCl_4 metabolism resulting from elevated CYP2E1 levels. Therefore, other factors such as fasting or diabetes that elevate CYP2E1 levels may also influence individual susceptibility to CCl_4 exposure.

Clotrimazole decreased CCl_4 metabolism 23% in the human liver microsome sample that exhibited the least (36%) suppression by CYP2E1 antibodies at 530 μM CCl_4, suggesting that CYP3A may contribute to CCl_4 metabolism in humans at high exposure levels. Since human CYP3A comprises approximately 30% and 70% of hepatic and intestinal total CYP450, respectively, this CYP450 could be important in CCl_4 metabolism and systemic uptake following high oral doses. CYP2B has been implicated in CCl_4 metabolism in rodents (Salmon *et al.*, 1985; Frank *et al.*, 1982); however, studies using expressed human CYP2B6 and inhibitory antibodies against CYP2B6 indicated that this form was unlikely to be important in CCl_4 metabolism in humans. It is interesting that the evidence which suggests that rodent CYP2B forms may be involved in CCl_4 metabolism and toxicity is primarily based on increased toxicity in animals pre-exposed to phenobarbital, an efficacious CYP2B inducer (Salmon *et al.*, 1985; Frank *et al.*, 1982). However, phenobarbital also induces CYP3A (Waxman *et al.*, 1992; Zangar *et al.*, 1998), so it is possible that the increased toxicity in phenobarbital pretreated animals was dependent on increased CYP3A rather than CYP2B expression.

Studies comparing the dose-related effects of CCl_4 inhalation exposures on hepatotoxic endpoints (i.e., necrosis, BrdU uptake and serum enzyme levels) have indicated that mice exhibit a toxic response at 20 ppm CCl_4, while hamsters and rats do not (Nikula *et al.*, 1998). In contrast, inhalation exposures at 100 ppm induced a much greater toxic response in hamsters than in mice (Nikula *et al.*, 1998). In our microsomal metabolism studies, we demonstrate that hamsters have a higher V_{max} than mice, although K_m values are similar (Table 2). Similarly, inhalation studies examining CCl_4 uptake in intact animals have shown that

hamsters exhibit a greater capacity for CCl_4 metabolism than do mice (Thrall *et al.*, 1999). Therefore, the greater hepatotoxic response observed in hamster at 100 ppm CCl_4 is most likely due to this species' greater capacity to metabolize CCl_4. However, based on microsomal metabolic parameters alone, the hamster should be at least as susceptible to CCl_4 hepatotoxicity as the mouse at the lower 20 ppm CCl_4 dose. These results suggest that factors in addition to these metabolic parameters are important in determining species differences in CCl_4 dose response. Therefore, these data suggest that a better understanding of the protective mechanisms present (or absent) in rodents and humans is necessary to extend rodent data to humans with confidence.

V. SPECIES DIFFERENCES IN THE TOXICOKINETICS OF INHALED CARBON TETRACHLORIDE

A. Background

Carbon tetrachloride is hepatotoxic and hepatocarcinogenic. The toxicokinetics and metabolism of CCl_4 have been studied in dogs (Robbins, 1929) and monkeys (McCollister *et al.*, 1951), but most commonly in rats using a variety of exposure routes, regimes and dose or exposure concentration ranges (Reynolds *et al.*, 1984; Paustenbach *et al.*, 1986a,b; Sanzgiri *et al.*, 1997; Yoshida *et al.*, 1999).

Carbon tetrachloride undergoes metabolism by cytochromes P4502E1 and 2B (Section III, this report) to form trichloromethyl and trichloromethylperoxyl free radicals, both highly reactive intermediates responsible for CCl_4's hepatotoxicity (McGregor and Lang, 1996). CCl_4 is either eliminated from the body unmetabolized, as the metabolites CO_2 and $CHCl_3$ in exhaled air, or as unidentified metabolites in urine and feces (Reynolds *et al.*, 1984: McCollister *et al.*, 1951). Rates of CCl_4 metabolism are species dependent (Sections III, IV, and VI, this report). Within in a given species, the extent or rate of CCl_4 metabolism depends on exposure route, dose or concentration and duration. High, bolus oral doses result in exhalation of a large percentage of the administered dose unchanged due to overwhelming the metabolic capacity of the liver, enzyme inhibition or hepatotoxicity (Reynolds *et al.*, 1984; Section III, this report). Lower bolus doses or gastrically infused doses are more efficiently metabolized (Reynolds *et al.*, 1984; Sanzgiri *et al.*, 1997). Following acute oral doses, whether low or high, the relative amounts of excretion of metabolites in exhaled air (CO_2 and $CHCl_3$) exceeded the amounts of metabolites excreted in urine and feces (Reynolds *et al.*, 1984).

The rates of metabolism of low CCl_4 concentrations of acutely inhaled CCl_4 were linear below 10 ppm (Yoshida *et al.*, 1999). At 10 ppm, the metabolic rate was 0.53 μmole/hour/kg body weight (BW), well below the maximum metabolic velocity for CCl_4 of 4.3 μmole/hour/kg reported by Paustenbach *et al.* (1988) for rats repeatedly inhaling 100 ppm CCl_4. That pathways of excretion are affected by exposure route and possibly duration is suggested by Paustenbach and co-workers (1986a,b; 1988), who report approximately 6, 9 and 84% of repeatedly inhaled CCl_4 was metabolized to CO_2, urinary and fecal metabolites, respectively.

Species differences in sensitivity to the hepatotoxic and hepatocarcinogenic effects of CCl_4 have been observed among rats, mice and hamsters repeatedly exposed by inhalation to 5–120 ppm CCl_4 (Section VII, this report; Taijira Matsushima, Director of Japan Bioassay Laboratory, personal communication). Rats were least sensitive, compared to mice and hamsters, to the hepatotoxic effect of repeatedly inhaled CCl_4 (Section VII) and notably less sensitive to the hepatocarcinogenic effects of chronically inhaled CCl_4 (Taijira Matsushima, personal communication). We hypothesize that species differences in the ability to metabolize CCl_4 are responsible for the observed differences in sensitivity among rats, mice and hamsters to the toxic effects associated with repeated CCl_4 inhalation exposure.

The toxicokinetics of inhaled CCl_4 in rats has been previously investigated (Sanzgiri *et al.*, 1997: Paustenbach *et al.*, 1986a,b), but high concentrations (100–1000 ppm), not relevant to human exposure (Threshold Limit Value of 5 ppm, ACGIH, 1993), were used. The toxicokinetics of inhaled CCl_4 has not previously been evaluated in mice or hamsters. The purpose of this study was to evaluate species differences in the toxicokinetics of 20 ppm CCl_4 inhaled once for 4 hours to test the above hypothesis and provide input into the revised PBPK model for CCl_4 described in Section VI of this report. The CCl_4 concentration used is four times greater than the current Threshold Limit Value for CCl_4. Repeated inhalation of 20 ppm for 4 and 13 weeks is sufficient to induce liver lesions in B6C3F_1 mice and Syrian hamsters (this report, Section VII) and produce hepatocellular adenomas and carcinomas in mice, but not rats, when inhaled for 2 years (Taijira Matsushima, personal communication). The results from these studies, in combination with the evaluations of species differences in the *in vitro* and *in vivo* whole-body metabolism of CCl_4 described in Sections III, IV, VI and VII of this report will provide information useful for developing an improved risk estimate for CCl_4.

B. Methods

1. Generator Solutions

^{14}C-labeled CCl_4 (9 mCi/mmole; 99.59% pure by HPLC) was purchased from Wizard Laboratories, Davis, CA. Carbon tetrachloride was purchased from Sigma Chemical Co., St. Louis, MO. Generator solutions containing ^{14}C-CCl_4 were prepared immediately before each exposure. The specific activity of the generator solutions was 0.039 μCi/μmole for rats and mice and 0.48 μCi/mmole for hamsters.

2. Animals

Male F344/Crl BR rats, $B6C3F_1$ mice and Syrian hamsters, purchased from Charles River Laboratories (Wilmington, WA), and approximately 12 weeks old at the time of exposure, were used. The animals were housed in shoe-box cages with hardwood chip bedding and filter caps. The animal rooms were maintained at 21 ± 1ºC with 20–60 percent relative humidity. There was a 12-hour light-dark cycle, with light beginning at 0600. Food (Harlan Teklad Certified Rodent Died [W], Harlan Teklad, Madison, WI) and water were provided *ad libitum*.

3. Exposure System

The inhalation exposure system consisted of a syringe pump, gas-tight Hamilton syringe, J tube and a 96-port brass nose-only exposure chamber, all enclosed in a glove box. CCl_4 was metered at an appropriate rate into the J tube, and the vapors were carried by a stream of N_2 into the chamber and mixed with chamber supply air. Chamber CCl_4 concentration was monitored using a Miran 1A infrared analyzer (Wilks Enterprises, South Norwalk, CT) with a monitoring wavelength of 12.6 μm.

4. Animal Exposures

Two, 4-hour inhalation exposures were conducted; one for rats and mice and another for hamsters. There were 20 animals of each species per exposure. At the end of the 4-hour exposure, four animals per species were immediately transferred to metabolism cages for collection of exhaled air, urine and feces. Metabolism collections were taken over a 48-hour

period after which the animals were sacrificed for tissue collection. Immediately after the exposure, and at 2, 6 and 24 hours after exposure, groups of four animals per species were sacrificed for tissue collection. Tissues taken at sacrifice included blood, lung, liver, kidney, brain and spleen.

5. Endpoints

a. Initial Body Burdens

Initial body burdens (IBBs) were determined using the animals held in metabolism cages and sacrificed 48 hours after exposure. The CCl_4 equivalents present in tissues, carcass and excreted by the rats, mice and hamsters were summed (by species) to provide an estimate of the IBB after exposure. The mean values for the four animals/species were used to calculate the percentage of the IBB (% IBB) in tissues and excreted as a function of time after exposure.

b. Pathways of Excretion

Urine, feces and exhaled CCl_4 and $^{14}CO_2$ were collected from the four animals of each species held in metabolism cages. Vessels used to collect urine and feces were kept on ice. Since urine and feces were not expected to contain CCl_4 or other volatile metabolites, no loss through volatilization was expected (Page and Carlson, 1994; McCollister *et al.*, 1951). Collections were made 6, 12, 24 and 48 hours after exposure. Charcoal traps were used for collection of CCl_4 and $CHCl_3$. Collections were made 1, 2, 3, 4, 6, 8, 12, 24 and 48 hours after exposure. Exhaled $^{14}CO_2$ was collected in KOH bubblers positioned after the charcoal traps at 6, 12, 18, 24 and 48 hours after exposure. After the 48-hour collection, the animals were euthanized by intraperitoneal injection of an overdose of pentobarbital. Blood was collected by cardiac puncture; lung, liver, kidneys, brain and spleen were removed and weighed. Tissues and carcasses were immediately frozen in liquid nitrogen and stored at –80ºC pending analysis.

c. Uptake, Distribution and Clearance

Immediately after exposure and 2, 6, 24 and 48 hours later, groups of four animals/species were sacrificed and tissues taken for analysis as described above.

6. Radioanalysis

Weighed samples of tissues and whole carcasses (without pelt) were digested using a 35 percent solution of tetraethyl ammonium hydroxide (Sachem, Austin, TX, 1 mL/g tissue). The weight of each digest was recorded. Weighed aliquots of digest were mixed with Ultima Gold XR scintillation cocktail (Packard Instrument Co., Meriden, CT) for quantitation of ^{14}C activity.

Urine samples were collected into preweighed vials and re-weighed after collection to determine the amount obtained. One-hundred µL aliquots were weighed into liquid scintillation vials and counted for ^{14}C activity. Each fecal sample was diluted and liquified with 1% Triton X100 (50:50 v/v). The total combined weight of each sample was recorded. Two hundred mg aliquots of each homogenate were weighed into tared vials and counted for ^{14}C activity.

Triplicate 2-g aliquots of charcoal were extracted by sonicating for 15 minutes with 10 mL dimethyl formamide at room temperature. The sonicates were centrifuged for 30 minutes at 1700 rpm to sediment the charcoal particles. Each extract was transferred to scintillation vials for quantitation of radioactivity. Three to five extracts were performed for each sample.

Immediately after each collection, the total volume of 2 N KOH in each bubbler used to trap $^{14}CO_2$ was recorded. One mL aliquots were counted for ^{14}C.

In all cases, aliquot counts for each sample were corrected for the total weight or volume of the corresponding original sample.

Radioactivity in samples was quantitated using a Packard Tri-Carb Model 2500TR liquid scintillation analyzer (Packard Instrument Co.). Quench correction was by the automatic external standard method. Samples were counted for 30 minutes. Minimum detectable activities for each sample type were calculated using the method of Altschuler and Pasternack (1963). The limit of detection was approximately 60 gross counts; limits of quantitation were approximately 140 gross counts.

7. Data Analysis and Statistical Evaluation

Group mean values, standard deviations and standard errors were calculated for all parameters measured. Data are presented as mean ± SEM values.

Statistical significance between tissue burdens among different species was compared using a one-way analysis of variance (ANOVA) with a Bonferroni post evaluation, adjusting for multiple comparisons using InStat statistical software (Graph Pad Software, Inc., San Diego, CA).

The clearance of CCl_4 equivalents from tissue was modeled as follows. The ^{14}C-CCl_4 equivalents in each tissue (as a percent of the IBB) were fit using a single component negative exponential equation, which incorporates a constant indicating a portion of the tissue burden was not undergoing clearance, or a two component negative exponential equation. Differences in the clearance parameters for each tissue were evaluated using Wald statistics (Gallant, 1975). The significance level was set at $p < 0.05$, adjusted for multiple comparisons.

C. Results

The IBBs present in rats, mice and hamsters at the end of a 4-hour exposure to 20 ppm CCl_4 are compared in Table 3. On a per kilogram BW basis, mice received the greatest and hamsters received the least dose of CCl_4.

The concentrations of CCl_4 equivalents in tissues at the completion of exposures and 48 hours later are compared in Table 4. Immediately after the exposure, concentrations of CCl_4 equivalents were highest in fat for rats and mice, with the next highest concentration present in liver. The reverse was true for hamsters. The ranking of species by concentration of CCl_4 equivalents in liver both at the completion and 48 hours after exposure was mouse > hamster > rat.

The distribution of CCl_4 equivalents as a % IBB in tissues other than fat immediately after and 48 hours after completion of exposure are compared in Figures 10 and 11, respectively. Both immediately after and 48 hours after exposure, the liver of all species contained the greatest % IBB with the greatest % IBB present in hamster liver, and the least in rat liver. Kidneys contained the next highest % IBB in all species at both time points.

For the most part, CCl_4 equivalents cleared from tissues very rapidly. Clearance kinetics of CCl_4 equivalents was tissue and species dependent (Tables 5–10). Clearance from blood of all species was described by a single component negative exponential function (Table 5) and was significantly faster for rats than for mice and hamsters. For most tissues (lung, liver, kidney, brain and fat), particularly for mice and hamsters, clearance was described by single

component negative exponential functions incorporating a constant, suggesting a binding of CCl_4 metabolites or incorporation of end products of metabolism into biosynthetic pathways.

All species excreted volatile organic ^{14}C-labeled compound(s), presumably unmetabolized CCl_4 and $CHCl_3$ in exhaled air and CO_2, or as metabolites in urine and feces. For rats, the majority of the IBB was excreted by exhalation of $CCl_4/CHCl_3$ (Table 11). For mice and hamsters, fairly similar amounts (approximately 30% of the IBB) were excreted by exhalation of unmetabolized $CCl_4/CHCl_3$ and of CO_2. Urinary excretion of CCl_4 metabolites accounted for approximately 1 percent of the IBB for rats and mice, but at least 8% of the IBB for hamsters. Hamsters also excreted a larger percentage of the IBB in feces than did rats or mice.

Exhalation of $CCl_4/CHCl_3$ and CCl_4 metabolized to CO_2 were described by single component negative exponential functions for all species (Tables 12 and 13). Although the rat excreted the greatest fraction of the IBB of CCl_4 equivalents as volatile organic compounds in exhaled breath, the rate of clearance was significantly slower than the rate of exhalation of volatile organic compounds by mice and hamsters. The rate of excretion of CCl_4 metabolized to CO_2 was greatest for hamsters and least for mice although the fractions of the IBB excreted as CO_2 by the two species were similar.

D. Discussion

The purpose of this study was to evaluate species differences in the toxicokinetics of acutely inhaled CCl_4 at concentrations more closely representing levels of human exposure. Accumulated body burdens of CCl_4 equivalents following 4 hours of exposure to 20 ppm CCl_4 ranged from 26–50 μmole/kg BW. These burdens are below oral doses reported to be acutely hepatotoxic in rats (0.3 mmole/kg; Reynolds *et al.*, 1984) and well below the total absorbed dose of 179 mg CCl_4/kg in rats inhaling 1000 ppm for 2 hours (Sanzgiri *et al.*, 1997).

The extent of CCl_4 metabolism was greatest for hamsters, which excreted 58% of their IBB as CO_2 and metabolites in urine and feces, and least for rats who excreted only 25% of their IBB by these routes. Rats exhaled over 60% of their IBB as unmetabolized CCl_4 or $CHCl_3$, while hamsters exhaled only 30% of their IBB as CCl_4 or $CHCl_3$. The extent of metabolism by mice falls in between that of hamsters and rats. These results are consistent with the results of the *in vivo* metabolism studies (Section VI, this report), indicating that hamsters have a greater affinity (K_m) for CCl_4 and capacity (V_m) to metabolize CCl_4 than do rats. Mice have a greater

capacity to metabolize CCl_4 than rats. Since metabolism of CCl_4 to reactive radical species is necessary to produce toxicity, the greater ability of hamsters and mice to metabolize CCl_4 is consistent with the greater extent of toxicity in this species compared to that of the rat.

Except for fat in rat and mouse, concentrations of CCl_4 equivalents were highest in liver immediately and 48 hours after completion of exposure. Liver also contained the largest fraction of the absorbed burden (IBB) of CCl_4. Interestingly, the relative % IBB in liver was greatest for hamsters, the species with the greatest capacity to metabolize (CCl_4) and least for rats, the species with the least CCl_4 metabolizing capacity (Sections IV and VI, this report). Concentrations of CCl_4 in liver were highest for mouse and least for rats and paralleled the species sensitivity to the hepatotoxic effects of 20 ppm CCl_4 repeatedly inhaled by rats, mice and hamsters (Section VII, this report). Presuming physiological parameters such as blood flow and liver, blood partition coefficients for CCl_4 are similar for all species (Section VI, this report; Thrall and Kenny, 1996; Andersen *et al.*, 1987; Paustenbach *et al.*, 1988), the observed species differences in concentrations and fractions of CCl_4 equivalents in liver likely reflect species differences in capability of the liver to metabolize CCl_4 rather than species differences in distribution of parent compound to liver. Therefore, liver dose and relative species sensitivity correlate with metabolic capability.

Rates of clearance of CCl_4 equivalents from tissues were generally rapid and significantly faster than the clearance half-times of 300–700 minutes reported for rats exposed to 1000 ppm CCl_4 for 2 hours (Sanzgiri *et al.*, 1997). The occurrence of a "non-clearing" fraction in some tissues, more commonly in mice and hamsters may be due to binding of CCl_4 metabolites to DNA and nuclear proteins (McGregor and Lang, 1996) or possibly the incorporation of CCl_4 metabolic products into normal biosynthetic pathways. The reason why a "non-clearing" fraction was not identified in liver, but found in other tissues such as fat and brain is not clear.

The results of our toxicokinetic evaluation in rats differ from those of Paustenbach and co-workers (1986a,b) in that they found the greatest extent of metabolites excreted in feces, and the least excreted as CO_2. Reasons for these inconsistencies may lie in differences in study experimental design, with the major differences being in exposure concentration and exposure duration.

The results of our studies support the results of the species comparisons of the *in vitro* (Section III) and whole-body metabolism (Section VI) of CCl_4 in that, among the three

species investigated, the rat appears to have the least ability to metabolize CCl_4. These findings are consistent with the fact that rats are least sensitive to the toxic effects of inhaled CCl_4 (Section VII) and support the hypothesis that species sensitivity relates to the capacity of the species to metabolize CCl_4.

VI. COMPARATIVE METABOLISM OF CARBON TETRACHLORIDE IN RATS, MICE AND HAMSTERS USING GAS UPTAKE AND PBPK MODELING

A. Background

CCl_4 is a potent hepatotoxin, causing increased liver weight, lipid peroxidation, fatty infiltration and liver necrosis (Uemitsu and Nakayoski, 1984; Uemitsu *et al.*, 1986; Rechnagel and Glende, 1973). The hepatotoxicity of CCl_4 has been attributed to the production of free radical metabolites (Recknagel, 1983; Dianzani, 1987; Connor *et al.*, 1986; Ahmad *et al.*, 1987; Persson *et al.*, 1990), with the CYP450s involved in the metabolism rapidly destroyed during the metabolic process (Noguchi *et al.*, 1982a,b). Additionally, CCl_4 is classified as a probable human carcinogen (B2), based on development of hepatocellular carcinomas in rats, mice and hamsters (EPA, 1998). The EPA estimated that the oral slope factor of 0.13 mg/kg/day is calculated using a geometric mean of the unit risk from hamster, mouse (two strains) and rat data as the risk estimate. However, these species-specific unit risks vary by 2 orders of magnitude, from 3.4×10^{-5} μg/L (hamster) to 3.1×10^{-7} μg/L (rat). The carcinogenic linearized multistage inhalation unit risk of 1.5×10^{-5} (μg/m^3) is calculated from the oral exposure data.

The rate of metabolism of CCl_4 in humans has not been investigated, but some information is available from studies with rats. For example, Gargas *et al.* (1986) reported a single saturable metabolic pathway for CCl_4 in F344 rats, with Michaelis-Menten constants K_m and V_{max} of 0.25 mg/L and 0.4 mg/hour/kg BW, respectively, based on *in vivo* gas uptake studies. Paustenbach *et al.* (1988) also analyzed gas uptake curves from Sprague-Dawley rats and obtained a V_{max} of 0.65 mg/hour/kg BW. Uemitsu (1986) estimated the *in vivo* K_m and V_{max} for CCl_4 in Sprague-Dawley rats to be 0.3 mg/L and 2.7 mg/hour/kg BW, respectively, based on evaluation of uptake curves from nose-only inhalation exposures. More recently, a re-evaluation of gas uptake data from Evans *et al.* (1994) found a K_m and V_{max} of 1.16 mg/L and 0.32 mg/hour/kg BW for young (65–72 day old) F344 rats (Simmons *et al.*, 1999).

With the exception of the study by Uemitsu (1986), the reported Michaelis-Menten metabolic constants have been estimated using a PBPK model for CCl_4. A number of

PBPK models are available (Veng-Pedersen, 1984; Veng-Pedersen *et al.*, 1987; Uemitsu, 1986); however, the model by Paustenbach *et al.* (1988) for rats and monkeys differs by addressing the elimination of metabolites in all excretory routes. None of the previous PBPK models for CCl_4 contains species-dependent comparisons of metabolic constants for CCl_4. Kinetic information for laboratory species other than the rat is extremely limited.

The objective of the present studies was to evaluate the *in vivo* K_m and V_{max} for mice and hamsters, in comparison to rats, using a closed chamber gas uptake system. This *in vivo* metabolic information for multiple species can be used to improve the versatility of the CCl_4 PBPK model by incorporating specific data on the relative metabolic capacity of various species. Ultimately, the resulting data should serve to improve current human health risk estimates.

B. Methods

1. Gas Uptake System

The closed atmosphere exposure system was constructed as described by Gargas *et al.* (1986) with minor modifications. In brief, the system consists of a 9.0-L desiccator jar with gas inlet and outlet fittings fashioned into a ¼ inch thick stainless-steel lid. A silicone rubber gasket was fit between the glass rim of the desiccator and the stainless-steel lid, and the assembly was clamped in place using thumb-screw brackets placed around the perimeter. Preliminary studies conducted with an empty chamber found the non-specific loss of CCl_4 to be independent of concentration and less than 5%/hour, a rate equivalent to that described by Gargas *et al.* (1986). The chamber atmosphere was recirculated using a Bellows (Model MB-41, Metal Bellows Corp., Los Angeles, CA) stainless-steel metal pump at 2.0 L/minute. Carbon dioxide was removed with SodaSorb (W.R. Grace & Co., Atlanta, GA). Oxygen concentration in the chamber was maintained at 19–21% by slowly adding UHP O_2 when an audible O_2 alarm (Cole-Parmer, Vernon Hills, IL) signaled concentrations dropped below 20%. The pressure in the chamber was continually monitored and stayed constant throughout the experiments.

Each experiment used seven mice, five hamsters or three rats for each exposure concentration. CCl_4 was added as a liquid through a septum fitting 12 inches upstream of the chamber in a volume to achieve the desired initial chamber concentrations. The volumes of injected CCl_4 were sufficiently small that volatilization was rapid. Chamber atmosphere was monitored prior to addition of CCl_4 and up to 6 hours thereafter.

Atmospheric concentrations of CCl_4 in the chamber were determined by GC using a Hewlett-Packard Model 5890 Series II system. The GC used a hydrogen flame ionization detector with nitrogen as the carrier gas at 30 mL/minute. The column was a Restek DB624 30 m, 3.0 µm film thickness (Restek, Bellefonte, PA). The detector was operated at 25ºC and the injector at 80ºC; the final oven temperature was 180ºC. Under these conditions, CCl_4 had a retention time of approximately 2.1 minutes.

2. Partition Coefficients

Substrate:air partition coefficients were determined for saline, olive oil, mouse and hamster blood, and hamster liver, muscle and fat (Table 15) using the vial equilibration technique described by Sato and Nakajima (1979). Tissue:air partition coefficients for the mouse were assumed to be equal to those reported for the rat (Gargas *et al.*, 1986). Tissue:blood partition coefficients were calculated by dividing tissue:air partition coefficients by blood:air partition coefficients.

3. PBPK Model

PBPK models for CCl_4 have been described previously (Thrall and Kenny, 1996; Paustenbach *et al.*, 1988; Gargas *et al.*, 1986); model parameters are given in Table 16. Additional differential equations were added to the model code to account for the change in chemical concentration in the chamber atmosphere as described by Gargas *et al.* (1986):

$$\frac{dC_{CH}}{dt} = N \times \frac{Q_p}{V_{CH}} \times \left[\frac{C_{art}}{P_b} - C_{CH} \right] - K_L \times C_{CH}$$

where

C_{CH} is the chemical concentration in the chamber air (mg/L)

N is the number of animals in the chamber

Q_P is the alveolar ventilation (L/hour)

V_{CH} is the volume of the chamber adjusted for the volume of the animals, assuming 1 L/kg BW (L)

C_{art} is the arterial blood concentration (mg/L)

P_b is the blood:air partition coefficient (unit less)

K_L is the first-order nonspecific loss rate ($hour^{-1}$).

Metabolic parameters for rats, mice and hamsters were obtained by computer optimization of the gas uptake data as described (Gargas *et al.*, 1986). In brief, the PBPK model containing the appropriate partition coefficients and physiological parameters for each species was used to simultaneously predict the family of gas uptake data. A maximum likelihood search algorithm in SimuSolv (Mitchell and Gauthier Associates, Concord, MA) was used to vary values of the Michaelis-Menten constants K_m and V_{max} until a reasonable visual fit was achieved, which described all time course data with a single set of constants. Since all sets of curves were simultaneously optimized, no statistical evaluation of variability in the optimized values was possible.

C. Results

1. Partition Coefficients

Substrate:air partition coefficients for CCl_4 were measured in saline, olive oil, mouse blood, and hamster blood, liver, fat, and muscle (Table 15). The muscle was used to represent the slowly perfused tissue compartment, and the liver was used to represent both the liver and the richly perfused tissue compartments in the PBPK model. Measured hamster tissue:air partition coefficients did not differ significantly from those reported for the rat (Gargas *et al.*, 1986; Evans *et al.*, 1994). Olive oil was used for comparison with the values obtained for hamster fat tissue, as well as a comparison to previously reported values for olive oil. The partition coefficient for olive oil:air (387:73) compared well with previously reported values of 374:11 (Gargas *et al.*, 1986) and 343:47 (Uemitsu, 1986), thus confirming the technique in our laboratory.

2. Gas Uptake Studies

Closed recirculating chamber exposures of rats, mice and hamsters were conducted with a variety of initial exposure concentrations. Model predictions and experimental data are given in Figures 12 and 13. An optimal fit of the family of uptake curves for CCl_4 was obtained by adjusting the metabolic rate constants K_m and V_{max} for each species (Table 17). The metabolic constants used to describe the uptake curves for the rat were taken from Gargas *et al.* (1986). These constants provided good agreement with the data and were not further optimized. The ability to replicate the current data using the metabolic rate constants from the Gargas *et al.*

(1986) study affirms that the PBPK model and gas uptake system were operating consistent with that previously reported.

In comparison to the rat, the series of gas uptake curves for the mouse indicate that the mouse has a slightly higher capacity (higher V_{max}) and lower affinity (higher K_m) for metabolizing CCl_4 than the rat (Table 17). As with the rat, the optimal fit of the family of uptake curves was obtained using a single saturable metabolic pathway in the PBPK model to describe the metabolism of CCl_4 (Fig. 14).

The slope of the gas uptake curves for the hamster differs considerably from either the rat or the mouse, particularly at the mid- and low-initial exposure concentrations. The optimal fit of the family of uptake curves for the hamster indicates that the hamster has a higher capacity (higher V_{max}) and lower affinity (higher K_m) for CCl_4 metabolism than either of the other two species studied (Table 17). As determined for the mouse and rat, the hamster PBPK model described metabolism as occurring through a single saturable pathway. However, the hamster PBPK model tended to over-predict the low-exposure gas uptake data (40 ppm exposure) and under-predict the high-exposure data (1800 ppm exposure). Inclusion of two saturable pathways (high and low capacity, high and low affinity) to describe CCl_4 metabolism did not improve the model prediction and was not pursued further (data not shown).

In contrast to the other species, gas uptake studies with the hamster indicated that alveolar ventilation (QP) and cardiac output (QC) were higher than expected based on previous studies (Andersen *et al.*, 1987). In the current studies, the chamber clearance by the hamster is consistent with an allometric constant of 28 L/hour/kg BW, or a minute ventilation rate of 171 mL/minute for a 0.15 kg hamster. In comparison, the U.S. EPA document on Air Quality Criteria for Particulate Matter uses a minute ventilation rate of 57 mL/minute for a 0.134 kg Syrian hamster (EPA, 1996). Regardless, the higher allometric constant used in the PBPK model described here was necessary to provide a reasonable fit to the experimental data. Furthermore, visual observations of the animals during the conduct of the gas uptake studies revealed that hamsters experienced an initial short period of narcosis, followed by an excitation phase, thus altering respiratory dynamics. Thus, QP and QC in the hamster were calculated according to the equations:

$$QP_{hamster} = 28 \text{ L/hour } (BW)^{0.74}$$

$$QC_{hamster} = 28 \text{ L/hour } (BW)^{0.74}$$

The use of 0.74 as the exponent for BW is based on studies with anesthetized animals, as reported by Guyton (1947).

In a similar study involving gas uptake data for chloroform, Corley *et al.* (1990) found it necessary to incorporate equations describing the loss of chloroform-metabolizing capacity in the PBPK model in order to adequately describe the mouse uptake data. Because CCl_4 is metabolized in a suicide inactivation fashion, wherein the specific form of CYP450 metabolizing CCl_4 is destroyed during the metabolic process (Noguchi *et al.*, 1982a, b), the equations describing enzyme loss were incorporated into the hamster PBPK model in an attempt to obtain better model predictions. Computer optimizations conducted with these modifications, setting k_{resyn} equal to 0, suggested that the value of k_{loss} was insignificant (approximately 3×10^{-11}). While this in no way implies that the metabolic process is different than that previously described (Noguchi *et al.*, 1982a, b), it does suggest that for CCl_4 the technique of gas uptake is not appropriately sensitive to detect enzymatic loss under these experimental conditions. Therefore, the gas uptake data for the hamster was described without enzyme destruction in the PBPK model.

3. Model Predictions from *In Vivo* Inhalation Studies

Species differences in the distribution and elimination of CCl_4 predicted using the metabolic constants derived from the gas uptake data can be compared with data collected from animals exposed by nose-only inhalation to 20 ppm $^{14}CCl_4$ for 4 hours (Thrall *et al.*, 1999). In these toxicokinetic evaluations, the amounts of radioactivity were determined in blood, tissues, urine, feces and exhaled breath (as $^{14}CO_2$ using a KOH trap or as $^{14}CCl_4$ using a charcoal trap) at intervals up to 48-hours post exposure. Although these studies focused on tracking the distribution and elimination of radioactivity rather than determination of parent and/or metabolite concentrations, there are apparent trends in the resultant data that suggest consistency with species differences in metabolism. For example, Figure 15 illustrates that rats eliminate significantly less radioactivity (as % total body burden) associated with metabolism ($^{14}CO_2$ and elimination in urine/feces) and more radioactivity associated with the parent compound (charcoal trap) compared to either hamsters or mice.

A comparison of PBPK model-predicted and experimentally observed values for select parameters from the nose-only inhalation study in rats, mice and hamsters are given in Table 18. In general, a good description of the experimental data (total metabolized,

total exhaled as parent, total exhaled as CO_2, and IBB) was obtained using the PBPK models incorporating the metabolic parameters determined from gas uptake studies. The ratios of predicted to observed data were generally less than a factor of 2, and often very close to 1, particularly for the rat and mouse (Table 18). No attempts were made to improve the model fits by varying additional parameters.

D. Discussion

The metabolic constants calculated from the gas uptake studies using species-specific PBPK models indicate that, under similar exposure conditions, the hamster would be the most efficient in metabolizing CCl_4, followed by the mouse, and that rats would metabolize the least CCl_4. This estimation appears to hold true when comparing the data collected from animals exposed by nose-only inhalation to 20 ppm $^{14}CCl_4$ wherein less radioactivity was associated with metabolism (trapped as $^{14}CO_2$, eliminated in urine and feces) than either mice or hamsters (Thrall *et al.*, 1999). Further, Zangar *et al.* (Section IV, this report) compared the *in vitro* anaerobic rate constants (K_m and V_{max}) for CCl_4 metabolism using F344 rats, $B6C3F_1$ mice, Syrian hamsters, and human liver microsomes using a method described by Andersen *et al.* (1996). These *in vitro* data suggest that hamsters are more efficient at metabolizing CCl_4 compared to mice, rats or humans (Table 19, lines 9 and 11).

Since data are available from both *in vitro* studies in humans and rodents and *in vivo* rodent studies, the procedure of Reitz *et al.* (1989) can be used to estimate *in vivo* human metabolic rate constants (Table 19, last column). Briefly, normalized *in vivo* first-order rate constants were obtained by dividing the quotient of V_{max} and K_m optimized from the gas uptake data (Table 19, line 6) by the size of the liver for each species, to give values of 6.08×10^{-2}, 1.30×10^{-1} and 2.47×10^{-1} for rats, mice and hamsters, respectively (Table 19, line 7). The corresponding *in vitro* first-order rate constants (Table 19, line 12) are calculated in the same manner, after adjusting the V_{max}/K_m quotient by the yield of microsomal protein per gram liver in the various species. The last row of Table 19 shows that the *in vivo/in vitro* ratios agree quite well (1.40 for rats, 1.01 for mice and 1.70 for hamsters).

Due to the fact that the *in vivo/in vitro* ratios between species are similar, the human *in vivo/in vitro* ratio was assumed to be the average value of the ratios from rats, mice and hamsters (1.37). Therefore, the human *in vivo* metabolic first-order rate constant per gram of liver can be estimated using the *in vitro* first-order rate determined in human samples (Table 19,

line 12). The human *in vivo* estimate of V_{max} (1.49 mg/hour/kg BW) is subsequently obtained by assuming the *in vivo* K_m in humans is the same as the rat, as suggested from the *in vitro* measurements. The incorporation of these estimated human metabolic rates, along with human physiological parameters (Table 16) and partition coefficients (Paustenbach *et al.*, 1988) into the PBPK model provides very good agreement between the model simulations and data reported by Stewart *et al.* (1961) (Fig. 16).

Prior to the current study, no metabolic rate constants for CCl_4 were available for the mouse or hamster. Thus, the extrapolation of animal data to humans was necessarily based on unknown biologic factors. However, when the basic features are provided for estimating absorption, distribution and elimination of the parent CCl_4 from rats and humans, the PBPK model can be modified to incorporate specific data on the relative ability of various species to metabolically activate CCl_4. The incorporation of these metabolic parameters should serve to substantially modify the conservative body-weight-to-surface-area correction that is currently used in estimating human health risks.

VII. DEFINING THE MECHANISMS OF CARBON TETRACHLORIDE-INDUCED HEPATIC TUMORS IN RATS AND MICE

A. Background

Carbon tetrachloride is classified as a probable human carcinogen (IARC, 1979). Generally, cancer risk is estimated for humans by assuming that 1) humans are as sensitive as the most sensitive rodent species in a rodent cancer bioassay conducted using high doses of the chemical, and 2) all carcinogens act via a directly genotoxic mechanism. Based on these assumptions, a linear, nonthreshold dose-response relationship is assumed at low doses, and a linearized multistage model is used to estimate cancer risks for environmental levels of the chemical (Andersen, 1983; Crump, 1984). However, *in vivo-in vitro* animal models and biochemical assays to measure DNA repair in mouse and rat heptocytes have shown that hepatocellular DNA is not directly damaged by CCl_4 or its metabolites (Ikegwuonu and Mehendale, 1991; Doolittle *et al.*, 1987). Therefore, it is likely that CCl_4 is a nongenotoxic carcinogen that acts by producing cytolethality and regenerative cell proliferation, thereby increasing the mutation frequency. If the CCl_4-induced neoplastic response only occurs at high doses that induce cytolethality and regenerative proliferation, and if these responses are absent at

lower environmental levels, then use of the high-dose data to extrapolate to expected responses at environmental exposures would lack scientific justification.

Instead, a risk model with a threshold, used by the EPA for noncancer toxic effects, would be more appropriate (Butterworth *et al.*, 1995). If CCl_4-induced hepatocellular tumors are only produced secondary to cytotoxicity, cytolethality and regenerative proliferation, then the shape of the dose-response curve and the no adverse effect level (NOAEL) for these essential effects should be defined. No increased risk of tumors would be expected at doses below the NOAEL. It is standard to extrapolate toxicity risk estimates from rodents to man, by applying an uncertainty factor. Because CCl_4 must be metabolized to CCl_3 by hepatic P450s to exert its cytotoxic effects, this uncertainty factor can be refined by comparing the metabolism of CCl_4 by rat, mouse, hamster and human hepatocytes. Metabolism and toxicokinetic data can be used to develop a PBPK model, and this model, along with clinical pathology, histopathology and cell proliferation data, can be used to predict a NOAEL level for human exposures to CCl_4.

The purposes of this study were to: 1) compare hepatic sensitivity for cell injury, death and regenerative cell proliferation among rats, mice and hamsters that repeatedly inhale or ingest CCl_4, and 2) determine whether inhalation exposure conditions ultimately resulting in liver tumors in rats and mice produce hepatic cell injury, death and regenerative cell proliferation under conditions of subchronic exposure.

B. Methods

1. Inhalation

Male F344/Crl BR rats, $B6C3F_1$ mice and Syrian hamsters were exposed in whole-body inhalation chambers to 0, 5, 20 or 100 ppm CCl_4 6 hours/day, 5 days/week. Groups of five to six animals per species per level were sacrificed after 1, 4 and 12 weeks of exposure, except there were 10 rats per level used for serum enzyme analyses at 12 weeks. On the Monday afternoon after exposure, preceding a Friday necropsy, an osmotic minipump containing BrdU in a solution of 0.5 N $NaHCO_3$ in saline was aseptically implanted in the dorsal throacolumbar subcutis of each animal while under halothane anesthesia. The concentration of BrdU loaded into the minipumps was adjusted so the dosing was 1 μg BrdU per gram BW per hour for all three species. At sacrifice, blood was obtained by cardiocentesis for serum sorbitol dehydrogenase (SDH) and alanine amino transferase (ALT) determinations. Livers were fixed in

4% buffered paraformaldehyde overnight, trimmed, processed and embedded in paraffin. Serial 5 μm sections of liver were placed on slides and 1) stained with hematoxylin and eosin (H&E) or 2) immunoreacted with antibody to BrdU to detect nuclei that incorporated BrdU during DNA synthesis (replicating cells) and counterstained with H&E. The point-counting method of planimetry was used to estimate the volume percent of the hepatic parenchyma that was necrotic. Microscopic fields within the H&E-stained sections were examined in a systematic, random fashion. Digitized images of the fields were captured using an Optronix color camera interfaced to an Olympus BH2-RFCA microscope and a Macintosh Quadra 950 computer. The images were projected on the computer monitor screen at a final magnification of 640X. A 64-point grid (Stereology Toolbox, Davis, CA) was superimposed over each image. The volume percent of the hepatic parenchyma that was necrotic was calculated for each animal from the number of points on necrotic hepatic parenchyma divided by total number of points on hepatic parenchyma $\times$ 100%. The sections immunoreacted with BrdU were used to measure cell replication in two ways. Fields were sampled at 600X final magnification in a systematic, random fashion using a light microscope fitted with an ocular counting grid. The BrdU-labeling index was calculated as the percentage of BrdU positive nuclei ([number of BrdU-labeled hepatocellular nuclei ÷ number of viable hepatocellular nuclei counted] $\times$ 100%). Also, the number of BrdU-labeled nuclei/mm^2 of liver was determined. Data were analyzed statistically using one-way analysis of variance and Student-Newman-Keuls Method ($p < 0.05$).

2. Ingestion

Male F344/Crl BR rats, B6C3F$_1$ mice, and Syrian hamsters were administered drinking water containing 0, 500 ppb or 5000 ppb CCl_4 *ad libitum* 7 days/week. Groups of five rats and hamsters and six mice per level were sacrificed after 1 or 4 weeks of exposure. On the Monday afternoon preceding a Friday necropsy, an osmotic minipump containing BrdU in a solution of 0.5 N $NaHCO_3$ in saline was aseptically implanted in the dorsal throacolumbar subcutis of each animal while under halothane anesthesia. The concentration of BrdU loaded into the minipumps was adjusted so the dosing was 1 μg BrdU per gram BW per hour for all three species. At sacrifice, blood was obtained by cardiocentesis for serum SDH and ALT determinations. Livers were fixed in 4% buffered paraformaldehyde overnight, trimmed, processed and embedded in paraffin. Serial 5 μm sections of liver were placed on slides and 1) stained with H&E or 2) immunoreacted with antibody to BrdU to detect nuclei that

incorporated BrdU during DNA synthesis (replicating cells) and counterstained with H&E. The point-counting method of planimetry was used to estimate the volume percent of the hepatic parenchyma that was necrotic. Microscopic fields within the H&E-stained sections were examined in a systematic, random fashion. Digitized images of the fields were captured using an Optronix color camera interfaced to an Olympus BH2-RFCA microscope and a Macintosh Quadra 950 computer. The images were projected on the computer monitor screen at a final magnification of 640X. A 64-point grid (Stereology Toolbox, Davis, CA) was superimposed over each image. The volume percent of the hepatic parenchyma that was necrotic was calculated for each animal from the number of points on necrotic hepatic parenchyma divided by total number of points on hepatic parenchyma × 100%. The sections immunoreacted with BrdU were used to measure cell replication in two ways. Fields were sampled at 600X final magnification in a systematic, random fashion using a light microscope fitted with an ocular counting grid. The BrdU-labeling index was calculated as the percentage of BrdU positive nuclei ([number of BrdU-labeled hepatocellular nuclei ÷ number of viable hepatocellular nuclei counted] × 100%). Also, the number of BrdU-labeled nuclei/mm^2 of liver was determined. Data were analyzed statistically using one-way analysis of variance and Student-Newman-Keuls Method ($p < 0.05$).

C. Results

1. Inhalation

a. Clinical Pathology (Fig. 17)

Results for ALT and SDH, enzymes that leak from damaged or necrotic hepatocytes, were generally similar. ALT and SDH were not significantly increased at any time in rats, mice or hamsters inhaling 5 ppm CCl_4, or in rats or hamsters inhaling 20 ppm CCl_4 compared to controls. ALT and SDH were significantly greater in rats exposed to 100 ppm CCl_4 compared to controls at 4 and 12 weeks. ALT and SDH were significantly greater in mice exposed to 20 and 100 ppm CCl_4 compared to controls at 1, 4 and 12 weeks. ALT was significantly greater in hamsters exposed to 100 ppm CCl_4 compared to controls at 1, 4 and 12 weeks. SDH was significantly greater in hamsters exposed to 100 ppm CCl_4 compared to controls at 1 and 12 weeks. The increases in ALT and SDH in hamsters were most striking at 1 week.

b. Hepatocellular Necrosis (Figure 18)

The volume percent of the hepatic parenchyma that was necrotic was not significantly increased at any time in rats, mice or hamsters inhaling 5 ppm CCl_4, or in rats or hamsters inhaling 20 ppm CCl_4 compared to controls. The percent of the hepatic parenchyma that was necrotic was significantly increased at 1, 4 and 12 weeks in rats exposed to 100 ppm CCl_4 compared to controls. The amount of necrosis increased progressively over time. The percent of the hepatic parenchyma that was necrotic was significantly increased at 1, 4 and 12 weeks in mice exposed to 20 and 100 ppm CCl_4 compared to controls. In mice, the amount of necrosis detected at sacrifice decreased from 1 to 12 weeks in those exposed to 20 ppm and was similar across all sacrifice times in those exposed to 100 ppm CCl_4. The percent of the hepatic parenchyma that was necrotic was significantly increased at 1, 4 and 12 weeks in hamsters exposed to 100 ppm CCl_4 compared to controls. In hamsters, the amount of necrosis detected at sacrifice decreased from 1 to 12 weeks in those exposed to 100 ppm CCl_4.

c. Hepatocellular Replication

BrdU-labeling indices (Fig. 19) were not significantly increased at any time in rats, mice or hamsters inhaling 5 ppm or in rats or hamsters inhaling 20 ppm CCl_4, or in rats inhaling 100 ppm CCl_4 compared to controls. The BrdU-labeling indices were significantly increased at 1, 4 and 12 weeks in mice exposed to 20 and 100 ppm CCl_4 compared to controls. At all times, the labeling indices were significantly greater in mice exposed to 100 ppm compared to 20 ppm. BrdU-labeling indices were significantly increased at 1, 4 and 12 weeks in hamsters exposed to 100 ppm CCl_4 compared to controls.

Results were similar when hepatocellular replication was estimated as the number of BrdU-positive hepatocellular nuclei per mm^2 of liver (Figure 20).

2. Ingestion

a. Clinical Pathology

There were no significant differences in serum ALT and SDH between control rats, mice and hamsters and rats, mice and hamsters that ingested CCl_4 at 1 or 4 weeks. The greatest differences in serum enzyme values between control and exposed animals were for ALT in hamsters exposed for 1 week. The mean values (± standard error) were 30.2

(± 2.4), 60.0 (± 17.2) and 53.8 (± 6.8) for controls, hamsters exposed to 500 ppb and hamsters exposed to 5000 ppb CCl_4, respectively ($p = 0.055$).

b. Hepatocellular Necrosis

There were no significant differences in the volume percent of necrotic hepatic parenchyma between control rats, mice and hamsters and rats, mice and hamsters that ingested CCl_4 at 1 or 4 weeks.

c. Hepatocellular Replication

BrdU-labeling indices and the number of labeled cells per mm^2 of liver were not significantly increased at any time in rats, mice or hamsters ingesting 500 ppb CCl_4 or in rats or mice ingesting 5000 ppb CCl_4. The BrdU-labeling index (Fig. 21) and labeled cells per mm^2 of liver were significantly increased compared to controls in hamsters ingesting 5000 ppb CCl_4 for 1 week. These indices were not increased in hamsters ingesting 5000 ppb CCl_4 for 4 weeks.

D. Discussion

1. Inhalation

The current ACGIH threshold limit value for CCl_4 is 5 ppm. No hepatocellular damage was detected by measurement of serum ALT and SDH, morphometric estimation of the volume percent of the hepatic parenchyma that was necrotic or analysis of hepatocellular replication in rats, mice or hamsters inhaling 5 ppm CCl_4 6 hours/day, 5 days/week, for 1, 4 or 12 weeks.

Hepatocellular damage occurred in mice, but not rats or hamsters, that inhaled 20 ppm CCl_4. Hepatocellular damage occurred in all three species at the 100 ppm CCl_4 exposure concentration. The increases in serum enzymes indicating hepatocellular damage, necrosis and cell proliferation were greater in hamsters than rats. These results show that mice are the most sensitive and rats the least sensitive to the hepatotoxic effects of inhaled CCl_4.

In hamsters that inhaled 100 ppm CCl_4, the hepatocellular necrosis was more severe at 1 week than at later times. This suggests that the hepatocytes remaining after the initial necrosis and the new hepatocytes formed as a result of replication are less sensitive to

CCl_4 than those present prior to exposure. Presumably, those hepatocytes that were most sensitive to CCl_4 were those with the greatest metabolic capability. The newly formed hepatocytes may be less differentiated or metabolically capable.

Hepatocellular proliferation did not occur in the absence of hepatocellular necrosis. Hepatocellular necrosis and regenerative cell proliferation occurred in mice at 20 and 100 ppm CCl_4, concentrations resulting in significantly increased numbers of hepatocellular adenomas and carcinomas in mice in a 2-year inhalation bioassay. Hepatocellular necrosis and a small, but not significant, increase in cell proliferation occurred in rats inhaling 100 ppm CCl_4, a concentration resulting in significantly increased numbers of hepatocellular adenomas and carcinomas in rats in a 2-year inhalation bioassay. The amount of necrosis in rats inhaling 100 ppm CCl_4 was greater at 12 weeks than at earlier times, suggesting that with continued exposure and necrosis, regenerative cell proliferation may have increased. Overall, these data support the hypothesis that CCl_4 induces tumors only at concentrations resulting in cell injury, death and regenerative proliferation.

2. Ingestion

No hepatocellular damage was detected by measurement of serum ALT and SDH, morphometric estimation of the volume percent of the hepatic parenchyma that was necrotic or analysis of hepatocellular replication in rats, mice or hamsters ingesting 500 ppb CCl_4 for 1 or 4 weeks. Hepatocellular damage was not detected in rats or mice ingesting 5000 ppb CCl_4 for 1 or 4 weeks or in hamsters ingesting 5000 ppb CCl_4 for 4 weeks. There was a significant increase in hepatocellular replication in hamsters that ingested 5000 ppb CCl_4 for 1 week. This increase in replication was most likely secondary to early hepatocellular damage. ALT was increased in exposed hamsters sacrificed after 1 week, but the increase at the time of sacrifice was not quite statistically significant. Most likely, most of the damage occurred in the first few days of exposure, and it is likely that serum enzymes indicating hepatocellular leakage would have been elevated if they had been measured earlier. It is difficult to make meaningful comparisons of serum concentrations of enzymes between enzymes (ALT versus SDH) and across species. The concentration of an enzyme in serum is dependent on both the magnitude and rate of enzyme release from tissue into blood and its subsequent removal from blood. The magnitude of enzyme release depends largely on the amount of cellular injury and the concentration of the enzyme in the cells. In most species, SDH is cleared very rapidly and has a

serum half-life after liver injury of minutes to hours. Thus, SDH tends to have diagnostic value during an initial tissue insult. Alanine amino transferase is also cleared rapidly, but its half-life tends to be hours to days. Thus, ALT generally has diagnostic value during and after an initial tissue insult. Although no specific data could be found for hamsters, minimal increases in serum ALT are often seen in dog serum with several conditions causing acute or low grade, chronic hepatic injury, while serum SDH is in the normal range due to its rapid clearance from blood (Hoffmann and Solter, 1999). Also, although positive correlations between the degree of necrosis and serum ALT activity have been observed in several species and by several investigators, other investigators have reported little histological evidence of necrosis in studies in which ALT activity was increased (Hoffmann and Solter, 1999). One explanation is that damaged hepatocytes may develop membrane blebs on the sinusoidal membrane. Membrane blebs can be shed and the membrane resealed as the blebs are pulled back through endothelial fenestrations. The blebs are released into the circulation and release cytoplasmic enzymes when they are degraded.

The technique of using minipumps to administer BrdU from Monday afternoon to the Friday sacrifice is referred to as continuous or cumulative labeling and is quite sensitive for detecting an increase in replication. Unlike single injections of BrdU, referred to as pulse labeling, the increase in replication does not have to occur right at the time of the pulse to be detected. The sensitivity of the labeling method used combined with the timing of the measurements explain why the serum enzymes might not be significantly elevated, although replication was elevated at the 1-week sacrifice. Overall, these data suggest that hamsters are more sensitive to the hepatotoxic effects of CCl_4 administered in drinking water than mice or rats.

VIII. RELEVANCE, IMPACT AND TECHNOLOGY TRANSFER

- The knowledge gained from our studies can be used to derive a new human health risk estimate for CCl_4 that could affect the level of cleanup required at contaminated sites like Hanford and thus save the DOE considerable amounts of money in CCl_4 cleanup efforts.
- While a revised health risk estimate for CCl_4 could save the DOE money in cleanup costs by reducing the level of cleanup required, the revised risk estimate must be performed by the EPA and will take considerable time. It is not known whether or when estimates will be revised and whether changes will be implemented in time to be of use to the DOE.

- The results of our studies will be used by risk assessors and by researchers interested in halogenated hydrocarbon toxicology and metabolism, and in species differences in sensitivity/resistance to toxic chemicals.
- The scientific capabilities of scientists at PNNL have been improved through construction and validation of a system to measure whole-body metabolism.
- This research has provided new insight in the mechanism of CCl_4-induced hepatic tumors. It has also provided new estimates for human metabolic constants for CCl_4 metabolism and evidence suggesting that humans, like rats, may be relatively resistant to the toxic effects of CCl_4.
- A major hurdle lies in having these new data used to develop a revised risk estimate for CCl_4.
- Individuals at Oak Ridge National Laboratory have expressed an interest in this work.

IX. PROJECT PRODUCTIVITY

The project accomplished the major portion of the proposed goals on schedule. The project design was revised to investigate the toxicokinetics of CCl_4 at only one exposure level (20 ppm). It was felt that studying the toxicokinetics of CCl_4 at 5 ppm (the current TLV) would be difficult because the inhaled material would clear too quickly and because investigation of the toxicokinetics of CCl_4 at 100 ppm would not be relevant to human exposure that occurs at much lower concentrations. The effects of repeated inhalation on the toxicokinetics of inhaled CCl_4 were also not investigated due to lack of funds.

X. PERSONNEL SUPPORTED

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XI. PUBLICATIONS

See Appendix A.

XII. REFERENCES

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Table 1

Summary of Significant Effects of Repeated CCl_4 Exposure by Inhalation or Drinking Water on Hepatic Microsomal Protein Content or CYP450 Immunodetectable Protein Levels and Associated Catalytic Activities

					CYP2E1		CYP2B	
Exposure Route	Species	Exposure Duration	Dose (ppm)	μS prot.	Protein	Activity[a]	Protein	Activity[b]
Inhalation	Rat	5 day	5					
			20	↑[c]	↑		↑	
			100	↑			↑	↓
		12 week	5					
			20		↑			
			100		↑	↓	↑	↓
	Mouse	5 day	5					
			20		↓	↓	↑	
			100		↓	↓	↓	↓
		12 week	5		↑			
			20		↑		↓	↓
			100			↓	↓	↓
	Hamster	5 day	5				↑	
			20				↓	↓
			100	↓	↓	↓	↓	↓
		12 week	5		↑			
			20		↑		↓	↓
			100		↓	↓	↓	↓
Drinking Water	Rat	1 week	0.5		↑	↑		
			5		↑	↑		
		4 week	0.5					
			5				↑	
	Mouse	1 week	0.5		↑			
			5		↑			
		4 week	0.5					
			5					
	Hamster	1 week	0.5	↑	↑		↑	
			5	↑	↑		↑	
		4 week	0.5		↑		↑	
			5		↑		↑	

[a]CYP2E1-associated chlorzoxazone 6-hydroxylase activity.

[b]Either or both of two CYP2B-associated activities, benzyloxyresorufin or ethoxytrifluorocoumarin O-deethylases.

[c]Arrows indicate that this parameter was significantly increased (↑) or decreased (↓) relative to samples from untreated animals.

Table 2

CCl_4 Metabolic Rate Constants from Human, Rat, Mouse and Hamster Microsomes

Species	K_m (μM)	V_{max} (nmole/minute/mg prot.)	R^2
Human	56.8[a]	2.26[b]	0.99[c]
Rat	59.1	3.10	0.98
Mouse	29.3	2.86	0.97
Hamster	30.2	4.10	0.99

[a]Each value represents the K_m derived from the appropriate curve shown in Figure 1, as calculated using SigmaPlot software.
[b]Each value represents the V_{max} derived from the appropriate line shown in Figure 1, as calculated using SigmaPlot software.
[c]Each value represents the correlation coefficient for the appropriate line and associated data points shown in Figure 1, as calculated using SigmaPlot software.

Table 3

^{14}C Initial Body Burdens[a]

Species	Initial Body Burden	
	μmole CCl_4/animal	μmole/kg
Rat	12.1 ± 0.25	50 ± 1.0
Mouse	1.97 ± 0.24[b]	69 ± 8.3
Hamster	3.65 ± 0.18[b,c]	26 ± 1.1[b,c]

[a]Results are the mean ± SEM of three (hamster) to four (rat and mouse) values.
[b]Mean significantly different from that of rat, $p < 0.05$, corrected for multiple comparisons.
[c]Mean significantly different from that of mouse, $p < 0.05$, corrected for multiple comparisons.

Table 4

Concentrations of CCl_4 Equivalents in Tissues
Immediately After and 48 hours After Exposure

Species	nmole CCl_4 Equivalents/g		Tissue:Blood Concentration at T = 0
	T = 0[a]	T = 48 hr[a]	
Rat			
Blood	6.07 ± 0.54	ND[b]	1
Lung	35.7 ± 2.96	6.56 ± 0.58	5.88
Liver	81.2 ± 2.54	18.6 ± 1.22	2.28
Kidney	45.6 ± 1.45	9.13 ± 0.23	0.56
Brain	7.98 ± 0.08	0.68 ± 0.03	0.18
Spleen	8.78 ± 1.22	1.00 ± 0.35	1.10
Fat	164 ± 8.68	2.26 ± 0.33	18.7
Mouse			
Blood	19.7 ± 5.07	1.02 ± 1.02	1
Lung	40.6 ± 4.82	11.0 ± 1.73	2.06
Liver	231 ± 19	29.8 ± 1.09	5.69
Kidney	102 ± 3.27	21.5 ± 1.60	0.44
Brain	5.90 ± 0.37	1.40 ± 0.06	0.06
Spleen	100 ± 28.7	13.0 ± 13.0	17.0
Fat	156 ± 72	12.0 ± 3.75	1.56
Hamster			
Blood	7.97 ± 1.06	1.68 ± 0.21	1
Lung	10.3 ± 0.50	3.05 ± 0.17	1.29
Liver	111 ± 24.1	19.8 ± 2.21	10.8
Kidney	10.0 ± 1.02	2.64 ± 0.16	0.09
Brain	1.84 ± 0.15	0.37 ± 0.02	0.18
Spleen	5.80 ± 1.06	2.38 ± 0.36	3.15
Fat	38.2 ± 7.56	4.03 ± 0.63	6.59

[a]Results are the mean ± SEM of 3–4 values.
[b]None detected.

Table 5

Rates of Clearance of CCl_4 Equivalents from Blood[a]

Species	Fraction Cleared	Clearance Parameter	T½
Rat	1.0	0.387 (0.116–0.658)	1.79 (1.05–5.97)
Mouse	1.0	0.053[b] (0.30–0.075)	13.1 (9.24–23.1)
Hamster	1.0	0.030[b] (0.022–0.037)	23.1 (18.7–31.5)

[a]Clearance data were fit using a single component negative exponential equation, $y = ae^{-bx}$, where a = the y intercept and b = the clearance parameter. The clearance half-time (T ½) = $\frac{0.693}{b}$ in hours.

[b]Value is significantly different from that for rat.

Table 6

Rates of Clearance of CCl_4 Equivalents from Lung[a]

Species	Fraction Cleared	Clearance Parameter	T½	Fraction Not Cleared	% IBB Not Cleared
Rat	0.79	0.095 (0.055–0.135)	7.3 (5.13–12.6)	0.21	0.055
Mouse	0.79	0.040 (0.006–0.074)	17 (9.36–115)	0.21	0.060
Hamster	0.74	0.065 (0.027–0.103)	11 (6.73–25.7)	0.26	0.056

[a]Clearance data were fit using a single component negative exponential function incorporating a constant, indicating that a fraction of the IBB of CCl_4 in lung was not cleared. Fraction cleared = % IBB in lung undergoing clearance divided by the % IBB present in lung immediately after the exposure. T½ = clearance half-time in hours = $\frac{0.693}{\text{clearance parameter}}$

Table 7

Rates of Clearance of CCl_4 Equivalents from Liver[a]

Species	Short-Term Clearance			Long-Term Clearance		
	Fraction Cleared	Clearance Parameter	T½	Fraction Cleared	Clearance Parameter	T½
Rat	0.37	0.229 (0.029–0.429)	3.0 (1.62–23.9)	0.62	0.020 (0.013–0.028)	35 (24.8–53.3)
Mouse	0.57	0.279 (0.011–0.546)	2.5 (1.27–63)	0.43	0.021 (0.010–0.033)	33 (21.0–69.3)
Hamster	1.0	0.021 (0.013–0.029)	33 (23.9–53.3)	NA	NA	NA

[a]Clearance data were fit using a single component negative exponential function (hamsters) or a two-component negative exponential function (rats and mice). T½ = clearance half-time in hours. Fraction cleared = % IBB in liver undergoing short-term clearance divided by the total % IBB in liver at the end of exposure.

Table 8

Rates of Clearance of CCl_4 Equivalents from Kidney[a]

	Short-Term Clearance			Long-Term Clearance			No Clearance	
Species	Fraction Cleared	Clearance Parameter	T½	Fraction Cleared	Clearance Parameter	T½	Fraction Not Cleared	% IBB Not Cleared
Rat	0.51	0.283 (0.148–0.418)	2.4 (1.66–4.68)	0.49	0.018 (0.014–0.022)	38 (31.5–49.5)	NA	NA
Mouse	0.81	0.069 (0.04–0.094)	10 (7.37–15.4)	NA	NA	NA	0.19	0.397
Hamster	0.68	0.100 (0.031–0.169)	6.9 (4.10–22.3)	NA	NA	NA	0.32	0.079

[a]Clearance data were fit using a two component negative exponential equation (rats) or a single component negative exponential equation incorporating a constant, indicating that a fraction of the IBB of CCl_4 in kidney did not undergo clearance. Fraction cleared = % IBB in kidney undergoing rapid, slow or no clearance divided by the % IBB in kidney immediately after the exposure. T½ = clearance half-time in hours.

Table 9

Rates of Clearance of CCl_4 Equivalents from Brain[a]

	Short-Term Clearance			Long-Term Clearance			No Clearance	
Species	Fraction Cleared	Clearance Parameter	T½	Fraction Cleared	Clearance Parameter	T½	Fraction Not Cleared	% IBB Not Cleared
Rat	0.81	0.428 (0.306–0.550)	1.6 (1.26–2.26)	0.19	0.014 (0.006–0.022)	50 (31.5–115)	NA	NA
Mouse	0.70	0.469 (0.283–0.656)	1.5 (1.06–2.45)	NA	NA	NA	0.30	0.035
Hamster	0.80	0.188 (0.055–0.320)	3.7 (2.16–12.6)	NA	NA	NA	0.20	0.010

[a]Clearance data were fit using a two component negative exponential equation (rats) or a single component negative exponential equation incorporating a constant, indicating that a fraction of the IBB of CCl_4 in brain did not undergo clearance. Fraction cleared = % IBB in brain undergoing rapid, slow or no clearance divided by the % IBB in brain immediately after the exposure. T½ = clearance half-time in hours.

Table 10

Rates of Clearance of CCl_4 Equivalents from Fat[a]

	Short-Term Clearance			Long-Term Clearance			No Clearance
Species	Fraction Cleared	Clearance Parameter	T½	Fraction Cleared	Clearance Parameter	T½	µg/g Not Cleared
Rat	0.83	0.267 (0.193–0.340)	2.60 (2.0–3.6)	0.17	0.053 (0.033–0.072)	13.1 (9.6–21.0)	NA
Mouse	0.95	1.96 (1.40–2.52)	0.35 (0.28–0.50)	NA	NA	NA	7.8
Hamster	0.92	0.352 (0.275–0.430)	1.96 (1.61–2.52)	NA	NA	NA	3.88

[a]Clearance data were fit using a two component negative exponential equation (rats) or a single component negative exponential equation incorporating a constant, indicating that a fraction of the IBB of CCl_4 in fat did not undergo clearance. Fraction cleared = % IBB in fat undergoing rapid, slow or no clearance divided by the % IBB in fat immediately after the exposure. T½ = clearance half-time in hours.

Table 11

Rates of Clearance of CCl_4 Equivalents from Spleen[a]

Species	Fraction Cleared	Clearance Parameter	T½	Fraction Not Cleared	% IBB Not Cleared
Rat	0.82	0.323 (0.225–0.421)	2.14 (1.61–3.08)	0.18	0.006
Mouse	1.0	0.041 (0.015–0.067)	16.9 (10.5–46.2)	NA	NA
Hamster	1.0	0.007 (-0.002–0.160)	99 (43–∞)	NA	NA

[a]Clearance data were fit using a single component negative exponential equation with (mouse and hamsters) or without incorporation of a constant, signifying that a portion of the tissue burden did not undergo clearance.

Table 12

Comparative Routes of Excretion of CCl_4 and Metabolites
(% IBB Excreted in 48 hours)[a]

Species	Volatile Organic Exhaled Compounds	CO_2	Urine	Feces
Rat	61.0 ± 1.80	21.9 ± 1.03	1.01 ± 0.17	1.83 ± 0.45
Mouse	38.5 ± 9.40[b]	35.1 ± 2.87[b]	0.72 ± 0.04	0.85 ± 0.29
Hamster	29.9 ± 2.71[b]	35.3 ± 4.27[b]	8.18 ± 1.62[b,c,d]	14.8 ± 0.85[b,c,d]

[a]Results are the mean ± SEM of 3–4 values.
[b]Value is significantly different from the corresponding value for rats. Analysis of variance with Bonferroni correction for multiple comparisons.
[c]Value is significantly different from the value for mice. Analysis of variance with correction for multiple comparisons.
[d]Some cross-contamination of urine and feces occurred for hamsters.

Table 13

Rates of Clearance of CCl_4 Equivalents Excreted as Volatiles in Exhaled Air[a]

Species	Fraction Cleared	Clearance Parameter	T½
Rat	1.0	0.162 (0.148–0.176)	4.3 (3.9–4.7)
Mouse	1.0	0.867[b] (0.714–1.02)	0.80[b] (0.68–0.97)
Hamster	1.0	0.193[b,c] (0.189–0.198)	3.6[b,c] (3.5–3.7)

[a]The results are the mean ± standard error of 4 values.
[b]Value is significantly different from that for rat.
[c]Value is significantly different from that for mouse.

Table 14

Rates of Clearance of CCl_4 as CO_2[a]

Species	Fraction Cleared	Clearance Parameter	T½
Rat	1.0	0.094 (0.090–0.097)	7.4 (7.1–7.7)
Mouse	1.0	0.079[b] (0.075–0.083)	8.8[b] (8.3–9.2)
Hamster	1.0	0.131[b,c] (0.129–0.134)	5.3[b,c] (5.2–5.4)

[a]Results are the mean ± SEM of 3–4 values.
[b]Value is significantly different from that for rat.
[c]Value is significantly different from that for mouse.

Table 15

Substrate:Air Partition Coefficients for CCl_4

Substrate	Rat	Mouse	Hamster
Saline:Air	0.18 ± 0.03[a] 0.35 ± 0.03[b]	—	—
Olive Oil:Air	387 ± 73[a] 374 ± 11[b] 343 ± 47[c]	—	—
Blood:Air	4.52 ± 0.35[b] 4.11 ± 0.26[c] 5.49 ± 0.95[d]	7.83 ± 2.18[a]	3.19 ± 0.81[a]
Liver:Air	14.2 ± 1.0[b] 16.2 ± 1.8[d]	—	12.6 ± 2.34[a]
Muscle:Air	4.54 ± 0.59[b] 6.66 ± 1.70[d]	—	2.51 ± 0.84[a]
Fat:Air	359 ± 11[b] 281 ± 50[d]	—	232 ± 37[a]

[a]Present study (n = 4 to 6).
[b]Gargas *et al.*, 1986.
[c]Uemitsu, 1986.
[d]Evans *et al.*, 1994.

Table 16

Physiological Parameters for PBPK Model[a]

Parameter	Rat[a]	Mouse[b]	Hamster[c]	Human[d]
Body Weight (kg)	0.25	0.025	0.15	70
Cardiac Output (mL/minute)	90	30	115[e]	5800
Alveolar Ventilation (mL/minute)	90	30	115[e]	5800
Blood Flow (% cardiac output)				
Liver	25	24	25	25
Fat	4	5	4	6
Richly Perfused	51	52	51	51
Poorly Perfused	20	19	20	18
Tissue Volume (% body weight)				
Liver	4	4	4	4
Fat	8	4	8	10
Richly Perfused	5	5	5	5
Poorly Perfused	74	78	74	62

[a]Parameters from Thrall and Kenny (1996).
[b]Parameters from Andersen *et al.* (1987).
[c]Physiological parameters set equivalent to the rat.
[d]Parameters from Paustenbach *et al.* (1988).
[e]Alveolar ventilation and cardiac output based on gas uptake data.

Table 17

In Vivo Metabolic Rate Constants

Species	V_{maxC} (mg/hour/kg BW)	K_m (mg/L)
Rat	0.40	0.25
Mouse	0.79	0.46
Hamster	6.39	1.14

Table 18

Comparison of Predicted and Observed Values for Selected Parameters from Toxicokinetic Data Collected from Rats, Mice and Hamsters 48 hours Post-Exposure to a 4-hour Nose-Only Inhalation Exposure of 20 ppm $^{14}CCl_4$

Species	Parameter	Model[a]	Data[b]	Ratio (pred/obs)
Rat	Initial body burden	6.8	11.7	0.6
	Total amount trapped on KOH	2.8	2.7	1.0
	Total amount trapped on charcoal	4.1	7.4	0.6
	Total amount metabolized[c]	3.7	3.8	1.0
Mouse	Initial body burden	2.7	1.9	1.4
	Total amount trapped on KOH	0.95	0.69	1.38
	Total amount trapped on charcoal	0.94	0.76	1.24
	Total amount metabolized[c]	1.3	1.4	0.9
Hamster	Initial body burden	4.1	3.7	1.1
	Total amount trapped on KOH	3.0	1.3	2.3
	Total amount trapped on charcoal	1.2	1.1	1.1
	Total amount metabolized[c]	3.9	2.9	1.3

[a]μmole.
[b]μmole equivalents of CCl_4; data from Benson *et al.* (unpublished observations).
[c]Represents the sum of radioactivity (in μmole equivalents) in urine, feces and trapped on KOH (CO_2).

Table 19

Comparison of Metabolism from *In Vivo* and *In Vitro* Studies

	Rat	Mouse	Hamster	Human[a]
Body Weight (g)	250	25	150	70000
Liver Weight (g)	10	1.0	6.0	2800
V_{maxC} (mg/hour/kg BW)	0.40	0.79	6.39	1.49
V_{max} (mg/hour/animal)	0.15	5.97×10^{-2}	1.69	29.15
In vivo K_m (mg/L)	0.25	0.46	1.14	0.25[b]
In vivo V_{max}/K_m	6.08×10^{-1}	1.30×10^{-1}	1.48	116.61
In vivo V_{max}/K_m/g liver	6.08×10^{-2}	1.30×10^{-1}	2.47×10^{-1}	4.16×10^{-2}
mg protein/g liver	13.8[c]	21.9[c]	17.8[d]	12.8[c]
In vitro V_{max} (nmole/minute/mg protein)[e]	3.10	2.86	4.10	2.25
In vitro V_{max} (nmole/hour/g liver)	42.78	62.79	73.18	28.80
In vitro K_m (nmole/L)[e]	59100	29300	30200	56800
In vitro V_{max}/K_m/g liver	4.34×10^{-2}	1.29×10^{-1}	1.45×10^{-1}	3.04×10^{-2}
Ratio (*in vivo*/*in vitro*)	1.40	1.01	1.70	1.37[f]

[a]Metabolic rates in humans calculated from *in vivo* gas uptake metabolism studies and *in vitro* incubations of liver microsomes of rats, mice, hamsters, and human accident victims (see text).
[b]Assumed to be equivalent to rat based on *in vitro* K_m comparisons.
[c]From Reitz *et al.* (1996).
[d]Estimated based on the average of mouse and rat.
[e]Data from Zangar *et al.* (unpublished results).
[f]Value calculated as the average of the rat, mouse, and hamster ratios.

FIGURE LEGENDS

FIGURE 1. Changes in hepatic microsomal protein content following low-dose exposure to CCl_4. Rats, mice or hamsters were exposed to various doses of CCl_4 by inhalation or in the drinking water and microsomal protein levels determined. For rats and hamsters, the microsomal protein levels are approximately equivalent to mg of microsomal protein per gram of liver. Each column and crossbar represent the mean and standard deviation of five samples. * Values are significantly different ($p < 0.05$) from the untreated group of the same species and exposure regimen.

FIGURE 2. Changes in hepatic microsomal CYP2E1 and chlorzoxazone 6-hydroxylase (CHZ OHase) activity in rats, mice or hamsters exposed to various doses of CCl_4 by inhalation. Each column and crossbar represent the mean and standard deviation of five samples. * Values are significantly different ($p < 0.05$) from the untreated group of the same species and exposure regimen.

FIGURE 3. Changes in hepatic microsomal CYP2E1 and chlorzoxazone 6-hydroxylase (CHZ OHase) activity in rats, mice or hamsters exposed to various doses of CCl_4 in the drinking water. Each column and crossbar represent the mean and standard deviation of five samples. * Values are significantly different ($p < 0.05$) from the untreated group of the same species and exposure regimen.

FIGURE 4. Changes in hepatic microsomal CYP2B and 7-ethoxy-4-trifluoromethylcoumarin (EFCOD) and benzyloxyresorufin (BzROD) O-dealkylase activities in rats, mice or hamsters exposed to various doses of CCl_4 by inhalation. Each column and crossbar represent the mean and standard deviation of five samples. * Values are significantly different ($p < 0.05$) from the untreated group of the same species and exposure regimen.

FIGURE 5. Changes in hepatic microsomal CYP2B and 7-ethoxy-4-trifluoromethylcoumarin (EFCOD) and benzyloxyresorufin (BzROD) O-dealkylase activities in rats, mice or hamsters exposed to various doses of CCl_4 in the drinking water. Each column and crossbar represent the

mean and standard deviation of five samples. * Values are significantly different ($p < 0.05$) from the untreated group of the same species and exposure regimen.

FIGURE 6. Metabolism of CCl_4 in microsomes from humans, rats, mice and hamsters. Rates of $CHCl_3$ formation in microsomes were determined under anaerobic conditions as described in *Methods*. Each data point and crossbar represent the mean and SE, respectively, of three or four incubations.

FIGURE 7. Metabolism of CCl_4 by expressed human CYP2E1. Rate of $CHCl_3$ formation was determined under anaerobic conditions as described in *Methods*. Each data point and crossbar represent the mean and SE of three or four microsomal incubations. Inset shows the metabolic rate constants derived from the fitted curve.

FIGURE 8. Inhibition of microsomal CCl_4 metabolism by monoclonal CYP2E1 antibodies. Microsomes isolated from three human livers (HL1, HL2 and HL3) were incubated with 17 or 530 μM CCl_4 in the presence or absence of inhibitory CYP2E1 antibodies. The rate of $CHCl_3$ formation was determined as described in *Methods*. Each column and crossbar represent the mean and SE, respectively, of three or four microsomal incubations. Numbers represent the percent decrease (significantly different at $p < 0.05$) of $CHCl_3$ formation resulting from addition of CYP2E1 antibodies relative to $CHCl_3$ levels measured in microsomes from the same human liver sample and the same CCl_4 concentration.

FIGURE 9. Inhibition of microsomal CCl_4 metabolism by chemical inhibitors. Microsomes isolated from the human liver HL2 were incubated with 530 μM CCl_4 in the absence of xenobiotic treatment (CON), in the presence of 0.1% acetonitrile alone (ACN) or in combination with 10 μM α-naphthoflavone (ANF), sulfaphenazole (SFZ) or clotrimazole (CTZ). These latter three agents are selective inhibitors of CYP1A, CYP2C9 and CYP3A, respectively. The rate of $CHCl_3$ formation was determined as described in *Methods*. Each column and crossbar represent the mean and SE, respectively, of four microsomal incubations. The number represents the percent decrease (significantly different at $p < 0.05$) in $CHCl_3$ formation resulting from addition of CTZ relative to ACN microsomes.

FIGURE 10. Tissue distribution of CCl_4 equivalents at the termination of a 4-hour inhalation exposure. CCl_4 equivalents are expressed as a percentage of the IBB present at the end of the 4-hour exposure. [a] Mean value is significantly different from the value for rats, $p < 0.05$, adjusted for multiple comparisons. [b] Mean value is significantly different from the value for mice, $p < 0.05$, adjusted for multiple comparisons.

FIGURE 11. Tissue distribution of CCl_4 equivalents 48 hours after termination of a 4-hour inhalation exposure. CCl_4 equivalents are expressed as a percentage of the IBB present at the end of the 4-hour exposure. [a] Mean value is significantly different from the value for rats, $p < 0.05$, adjusted for multiple comparisons. [b] Mean value is significantly different from the value for hamsters, $p < 0.05$, adjusted for multiple comparisons.

FIGURE 12. The uptake of CCl_4 from a closed recirculated atmosphere by three naï ve F344 rats per exposure. The initial chamber concentrations were 3, 4, 40, 163, 316 and 1293 ppm. The smooth curves were generated by the PBPK model using the constants given in Table 2.

FIGURE 13. The uptake of CCL_4 from a closed recirculated atmosphere by seven naï ve $B6C3F_1$ mice per exposure. The initial chamber concentrations were 43, 167 and 1206 ppm. The smooth curves were generated by the PBPK model using the constants given in Table 2.

FIGURE 14. The uptake of CCl_4 from a closed recirculated atmosphere by five naï ve Syrian Golden hamsters per exposure. The initial chamber concentrations were 40, 188, 217 and 1819 ppm. The smooth curves were generated by the PBPK model using the constants given in Table 2.

FIGURE 15. Naï ve rats, mice and hamsters exposed to 20 ppm $^{14}CCl_4$ for 4 hours by nose-only inhalation. ^{14}C in tissues, urine, feces and breath (both charcoal and KOH trap) was determined at intervals up to 48 hours post exposure. Data represent the activity in each fraction as a percent of total recovered radioactivity. (* statistically differs from rat using a two-tailed t-test at $p = 0.05$; ** statistically differs from mouse using a two-tailed t-test at $p = 0.05$).

FIGURE 16. Comparison of the actual versus predicted concentration of CCl_4 in the expired breath of humans exposed to 10 ppm of CCl_4 for 180 minutes (data from Stewart *et al.*, 1961). Model simulations were conducted using human partition coefficients (Paustenbach *et al.*, 1988) and the V_{max} and K_m predicted here.

FIGURE 17. Serum alanine amino transferase in rats, mice and hamsters exposed to 0, 5, 20 or 100 ppm CCl_4 for 1, 4 or 12 weeks. n = five or six animals per exposure concentration per time, except there were 10 rats per group at 12 weeks.

FIGURE 18. Hepatic parenchymal necrosis estimated from point counting in rats, mice and hamsters exposed to 0, 5, 20 or 100 ppm CCl_4 for 1, 4 or 12 weeks. n = five or six animals per exposure concentration per time.

FIGURE 19. Hepatocellular replication expressed as BrdU-labeling indices in rats, mice and hamsters exposed to 0, 5, 20 or 100 ppm CCl_4 for 1, 4 or 12 weeks. n = five or six animals per exposure concentration per time.

FIGURE 20. Hepatocellular replication expressed as BrdU-labeled nuclei per unit area in rats, mice and hamsters exposed to 0, 5, 20 or 100 ppm CCl_4 for 1, 4 or 12 weeks. n = five or six animals per exposure concentration per time.

FIGURE 21. Hepatocellular replication expressed as BrdU-labeling indices in hamsters exposed to 0, 500 or 5000 ppb CCl_4 in drinking water for 1 or 4 weeks. n = five animals per exposure concentration per time.

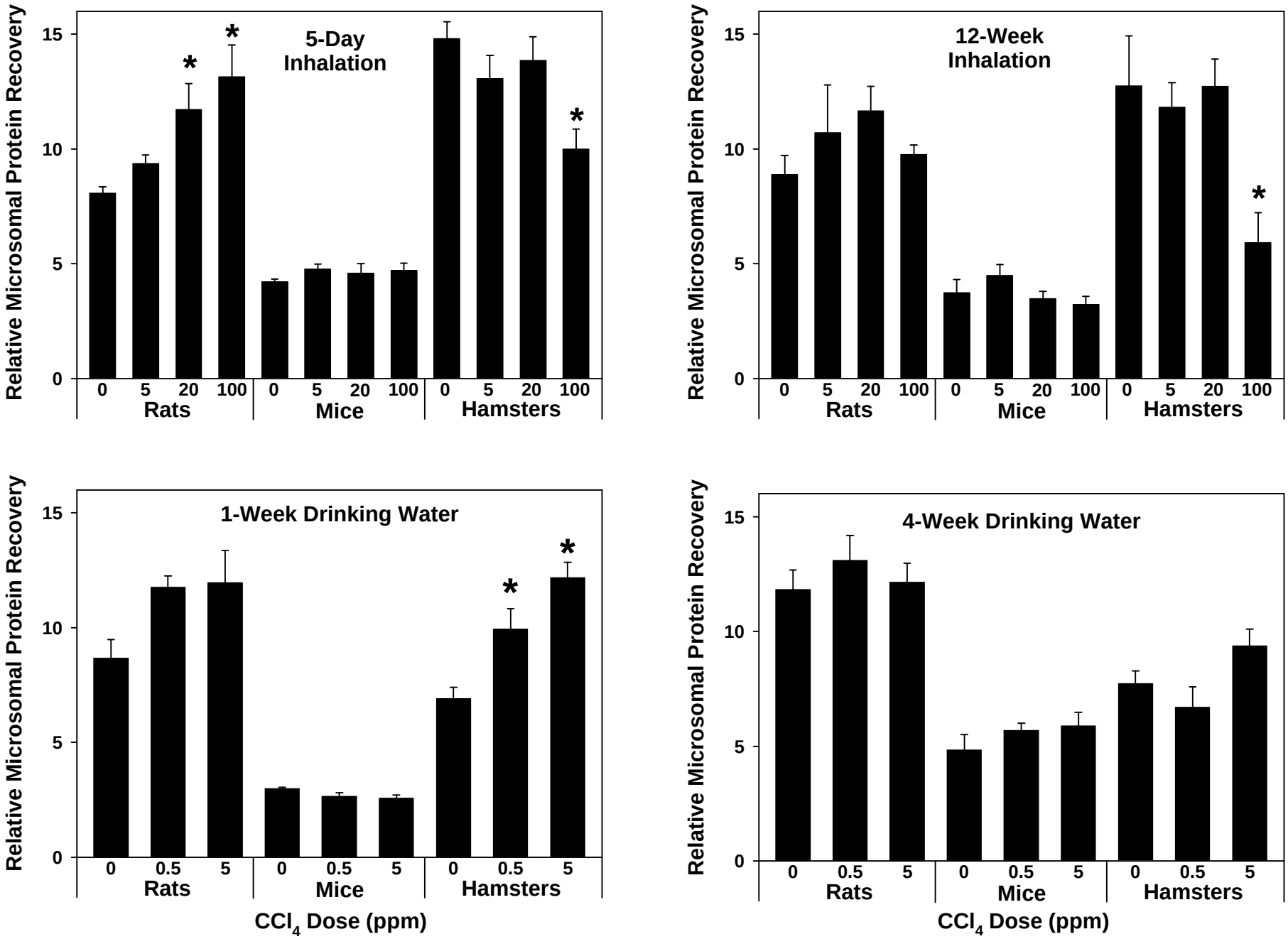
5-Day Inhalation
12-Week Inhalation
1-Week Drinking Water
4-Week Drinking Water
Relative Microsomal Protein Recovery
0
5
10
15
*
0
5
20
100
0.5
Rats
Mice
Hamsters
CCl4 Dose (ppm)

Figure 1

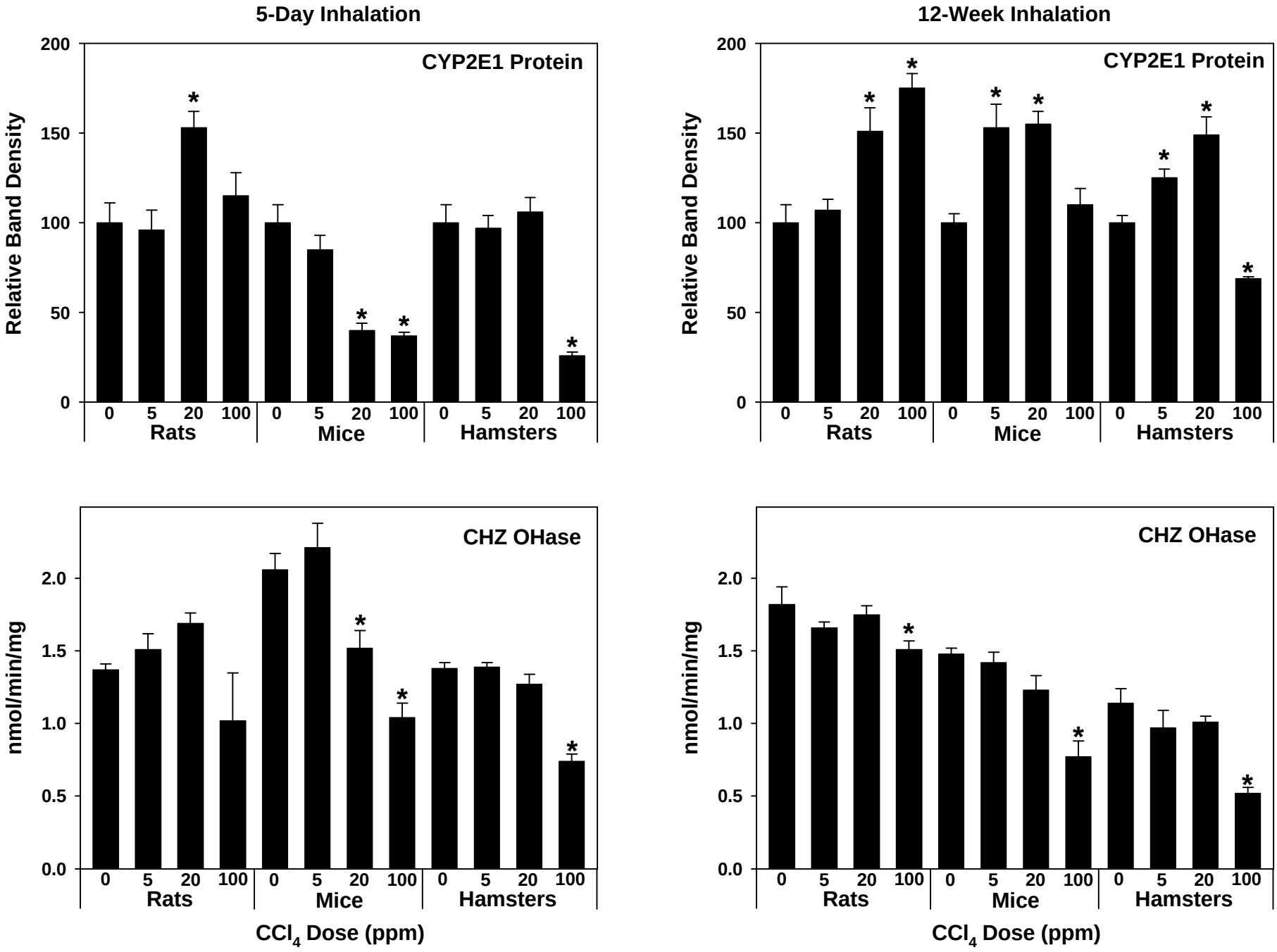
5-Day Inhalation
12-Week Inhalation
CYP2E1 Protein
CHZ OHase
Relative Band Density
nmol/min/mg
0
50
100
150
200
0.0
0.5
1.0
1.5
2.0
0 5 20 100
Rats
Mice
Hamsters
CCl4 Dose (ppm)

Figure 2

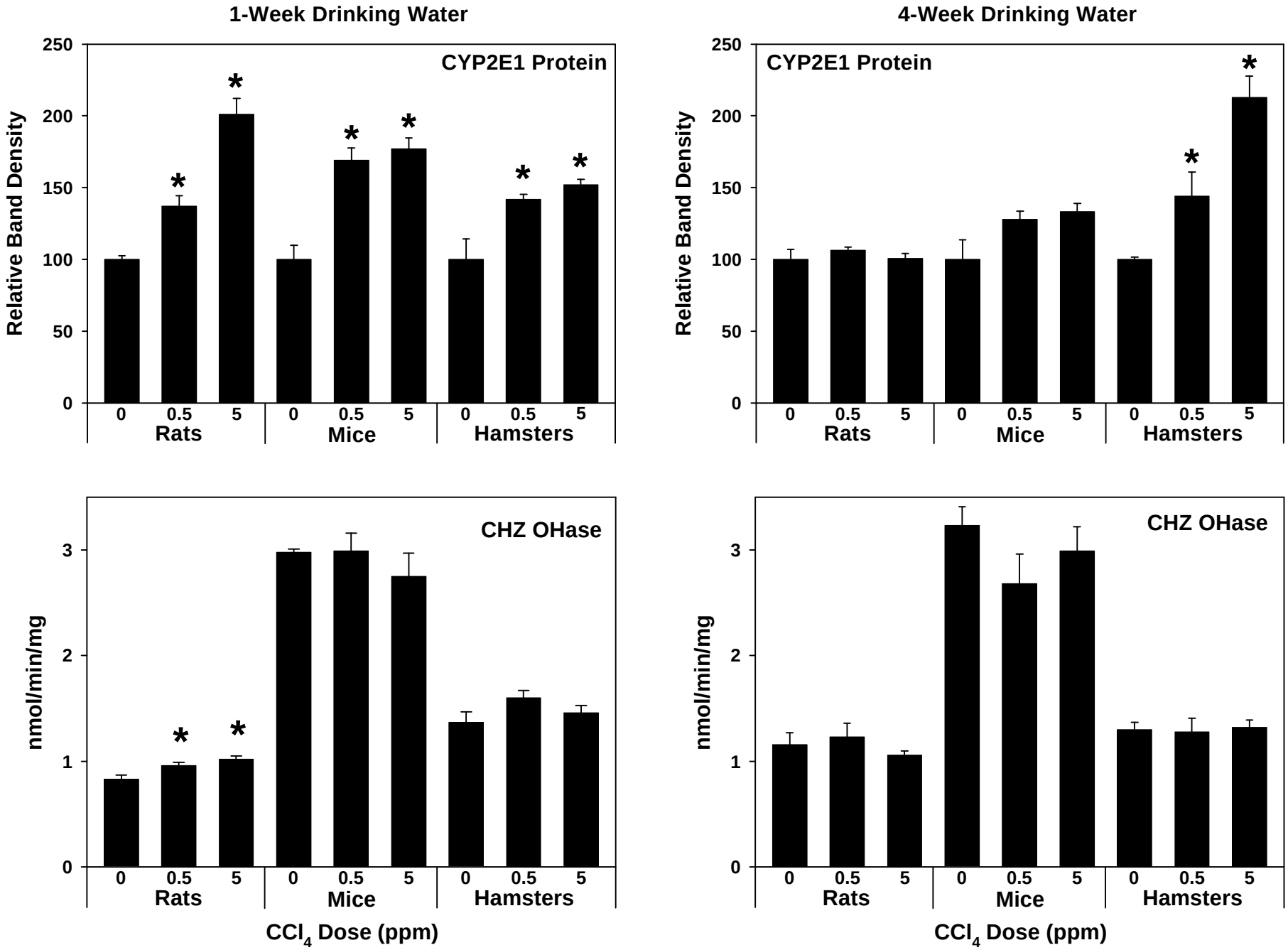
1-Week Drinking Water
4-Week Drinking Water
CYP2E1 Protein
Relative Band Density
250
200
150
100
50
0
0
0.5
5
Rats
Mice
Hamsters
*
CHZ OHase
nmol/min/mg
3
2
1
CCl_4 Dose (ppm)

Figure 3

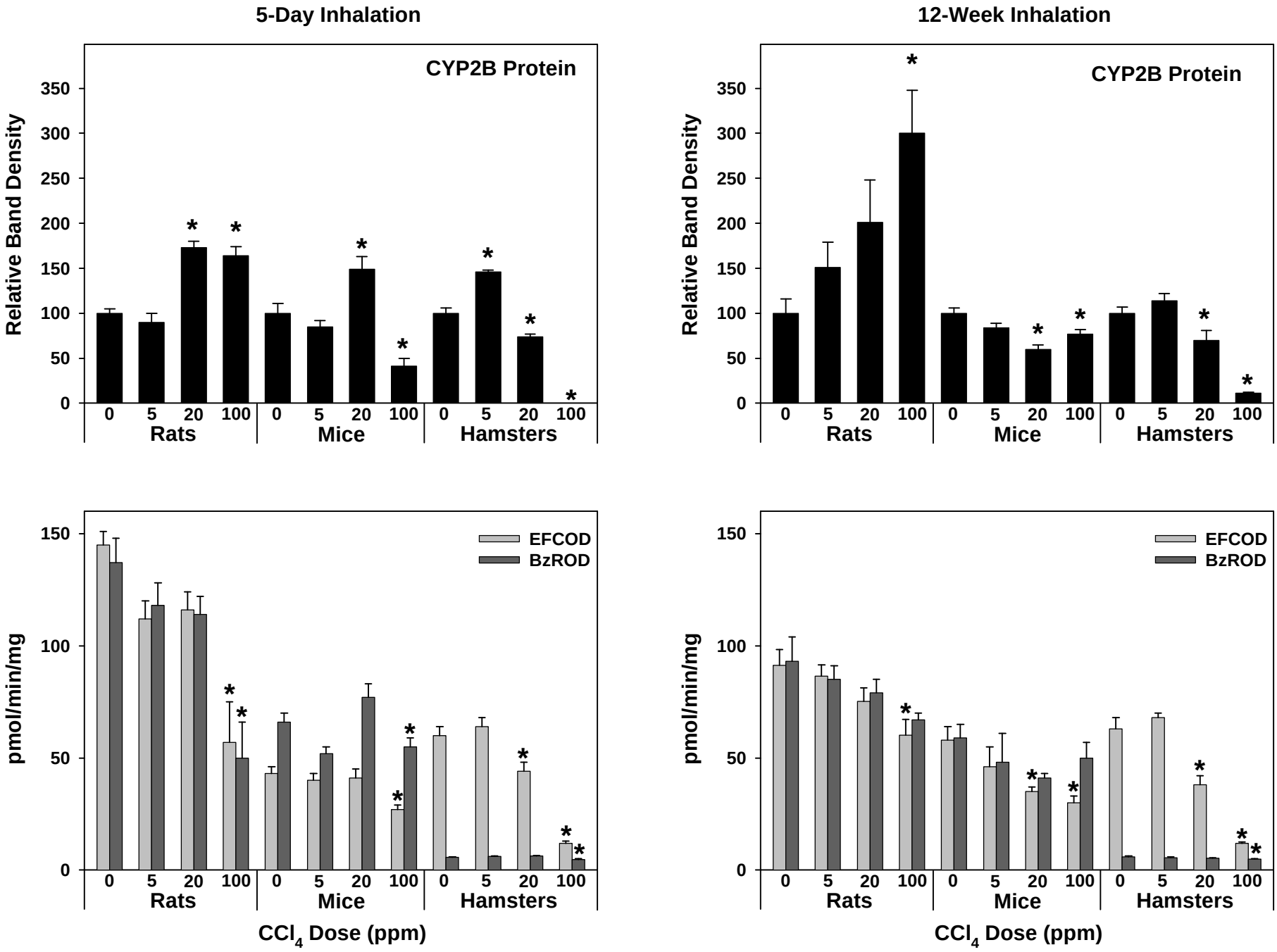
5-Day Inhalation
12-Week Inhalation
CYP2B Protein
Relative Band Density
0
50
100
150
200
250
300
350
0 5 20 100
Rats
Mice
Hamsters
EFCOD
BzROD
pmol/min/mg
CCl4 Dose (ppm)

Figure 4

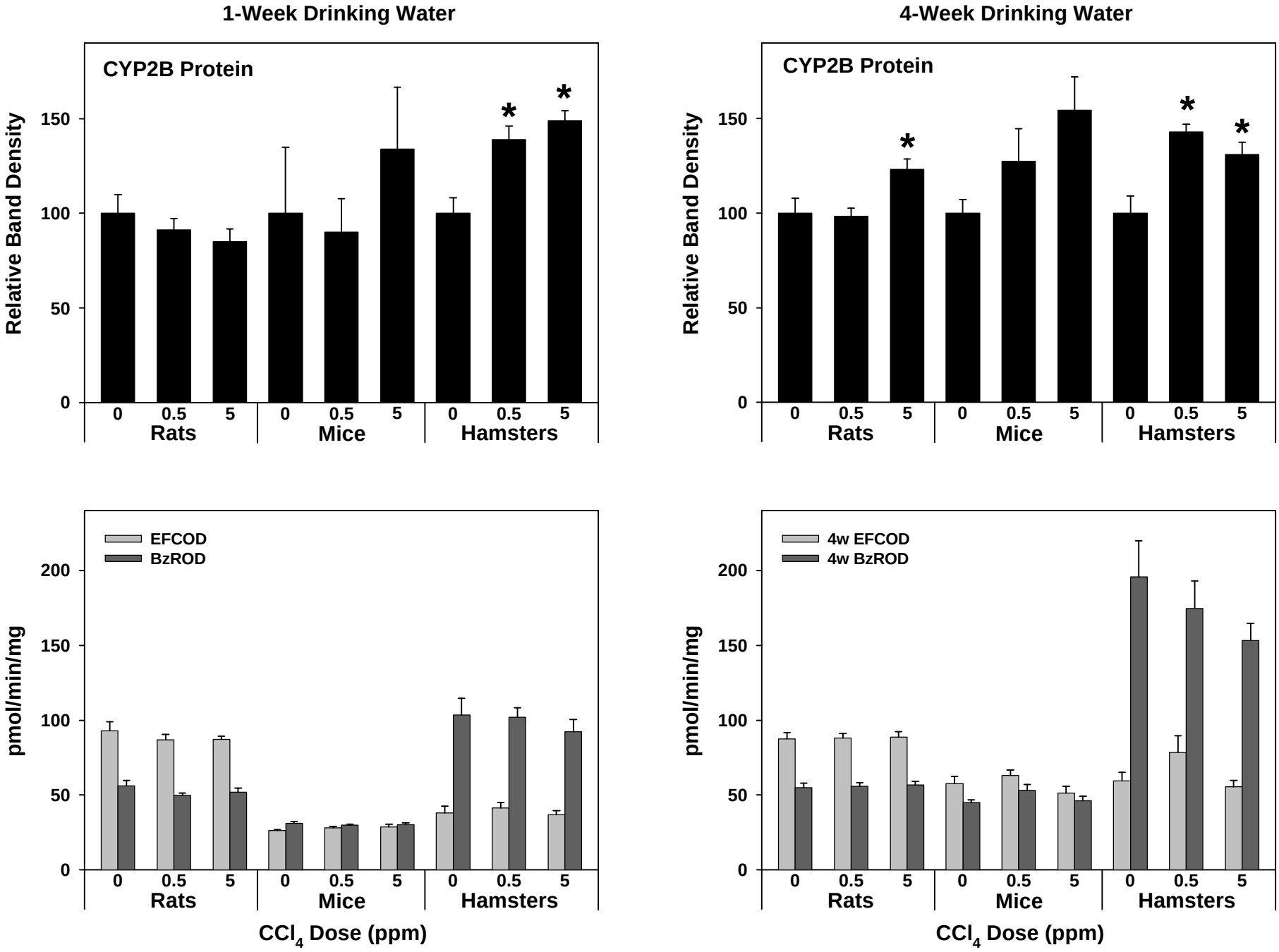
1-Week Drinking Water
4-Week Drinking Water
CYP2B Protein
Relative Band Density
0
50
100
150
*
0
0.5
5
Rats
Mice
Hamsters
EFCOD
BzROD
4w EFCOD
4w BzROD
pmol/min/mg
200
CCl4 Dose (ppm)

Figure 5

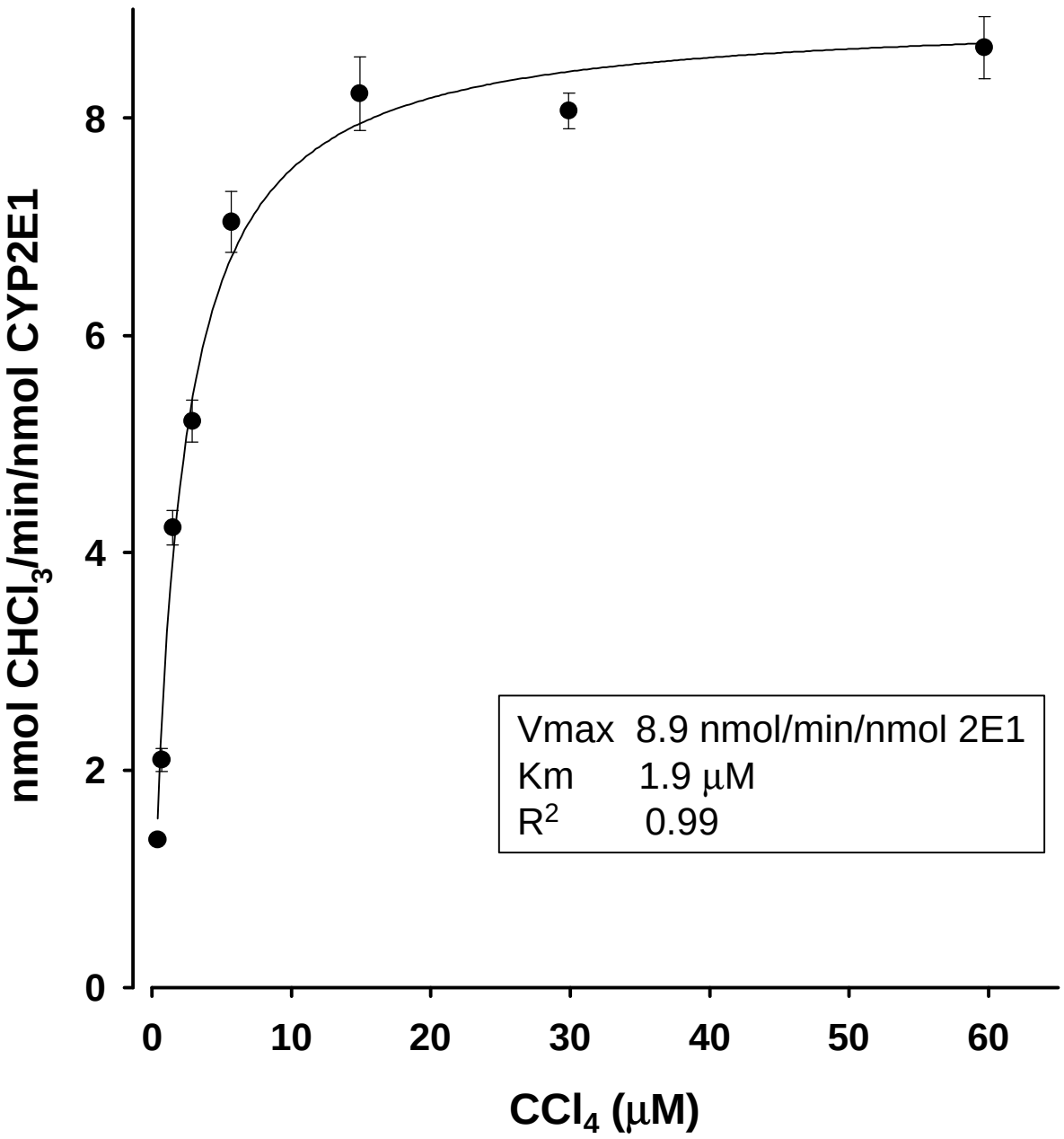
nmol CHCl3/min/nmol CYP2E1
0
2
4
6
8
0
10
20
30
40
50
60
CCl4 (μM)
Vmax 8.9 nmol/min/nmol 2E1
Km 1.9 μM
R2 0.99

Figure 6

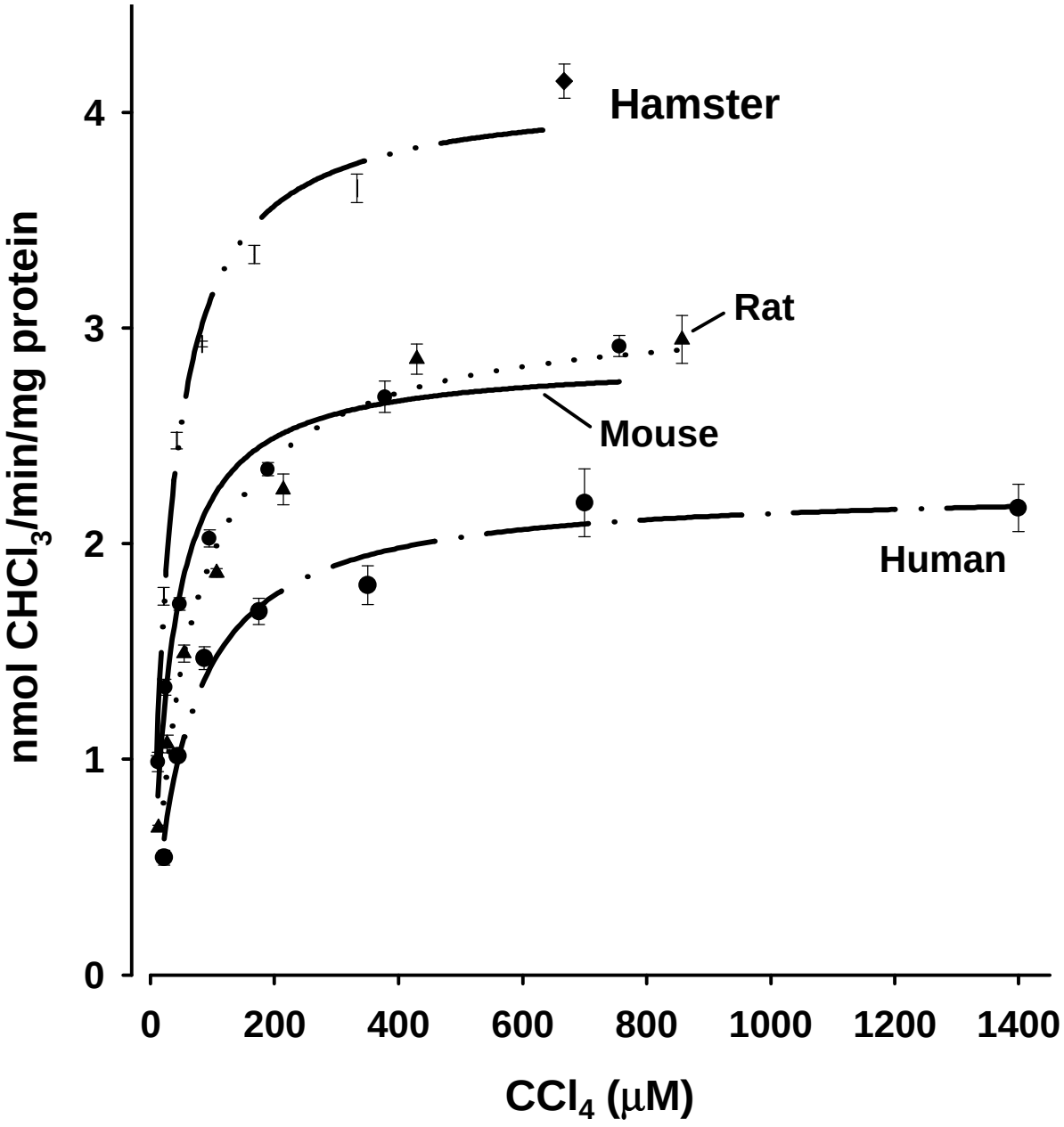
Hamster
Rat
Mouse
Human
nmol CHCl3/min/mg protein
CCl4 (μM)
0
1
2
3
4
0
200
400
600
800
1000
1200
1400

Figure 7

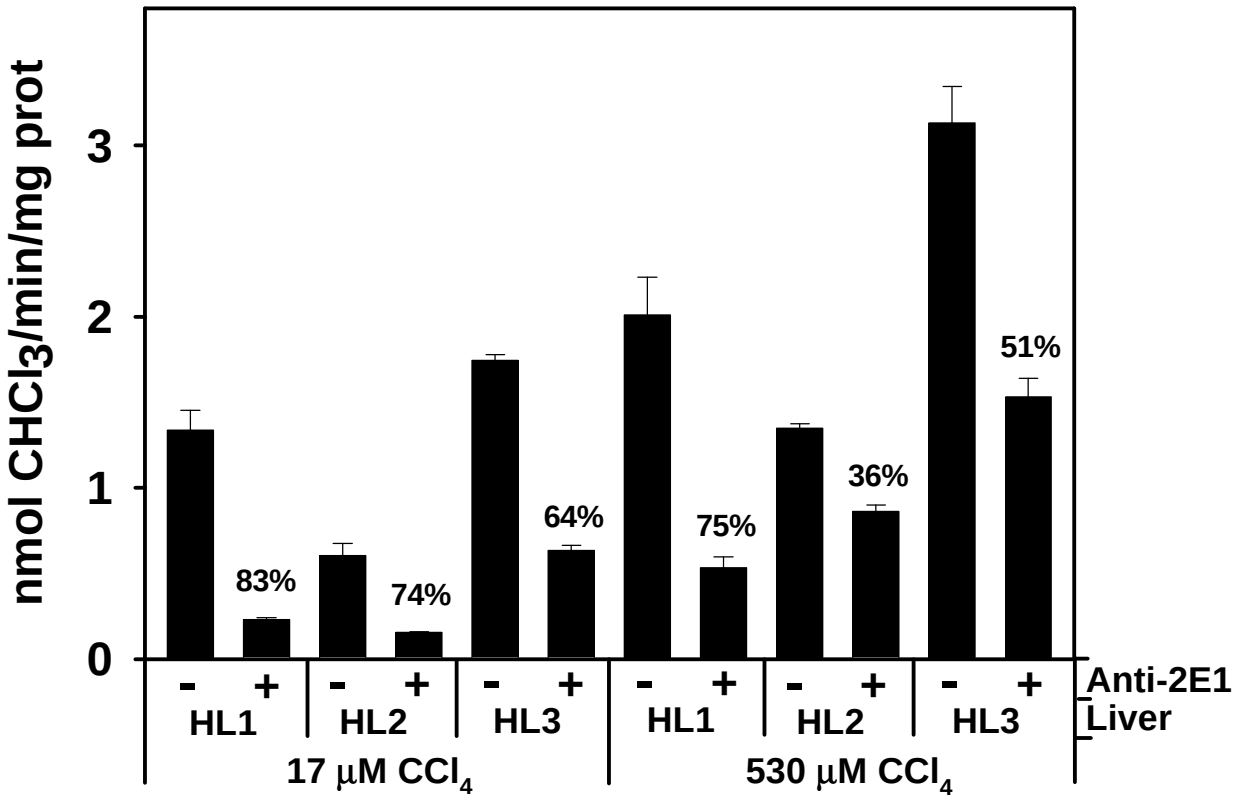
nmol CHCl3/min/mg prot
0
1
2
3
83%
74%
64%
75%
36%
51%
- + - + - + - + - + - +
HL1 HL2 HL3 HL1 HL2 HL3
17 μM CCl4
530 μM CCl4
Anti-2E1
Liver

Figure 8

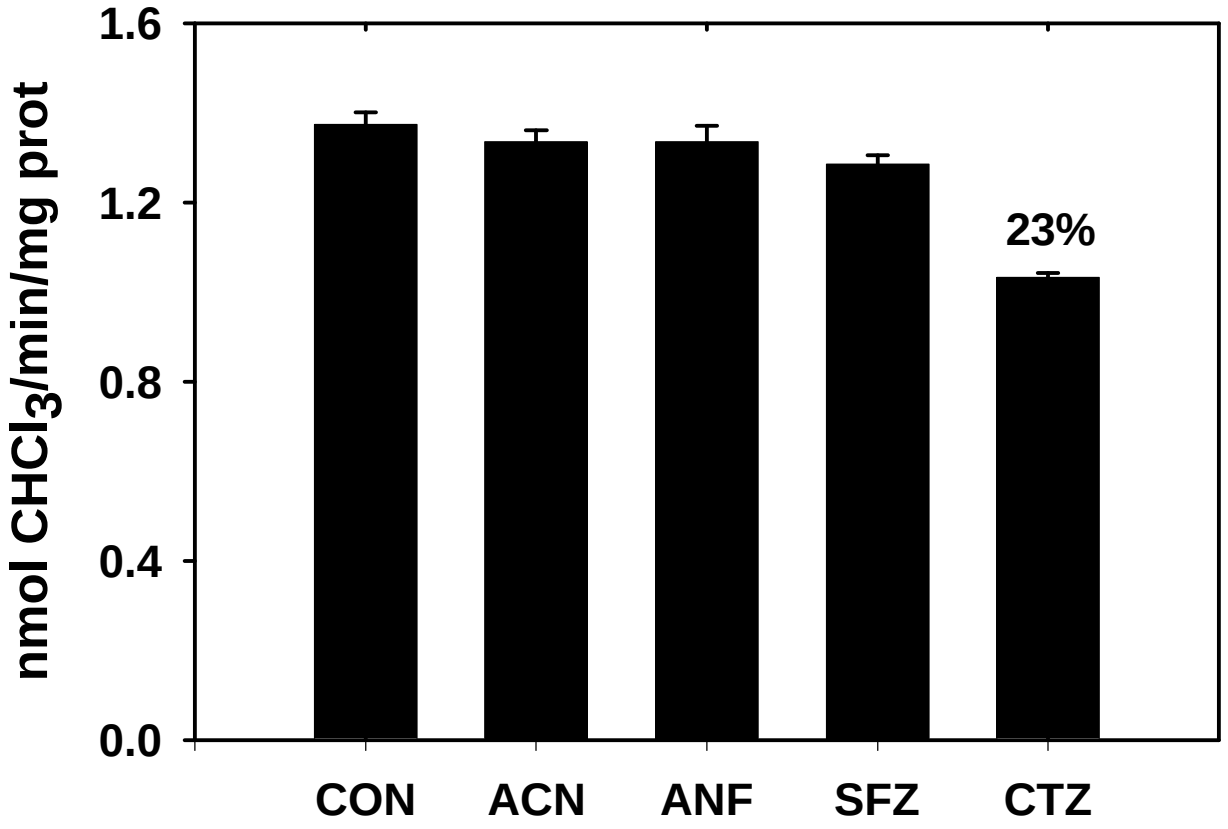

Figure 9

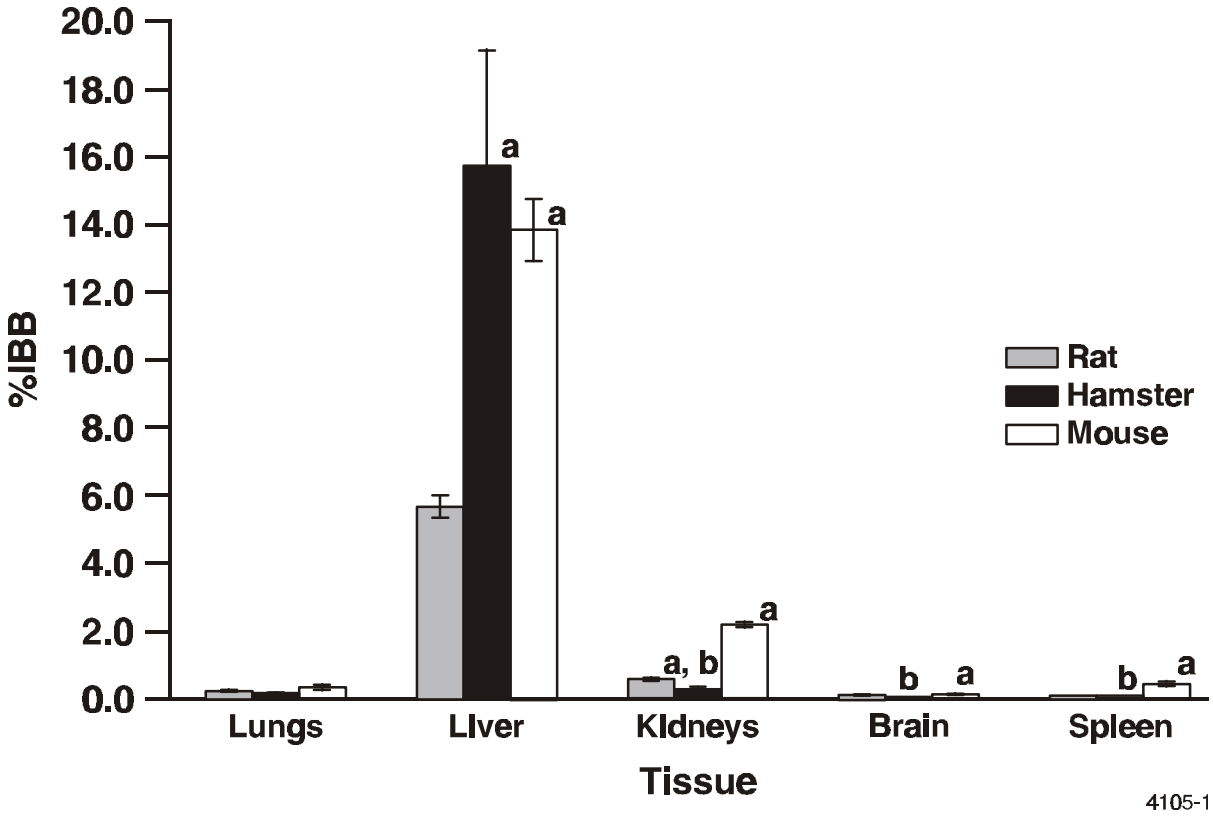
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
%IBB
a
a
a, b
a
b
a
b
a
Rat
Hamster
Mouse
Lungs
Liver
Kidneys
Brain
Spleen
Tissue
4105-1

Figure 10

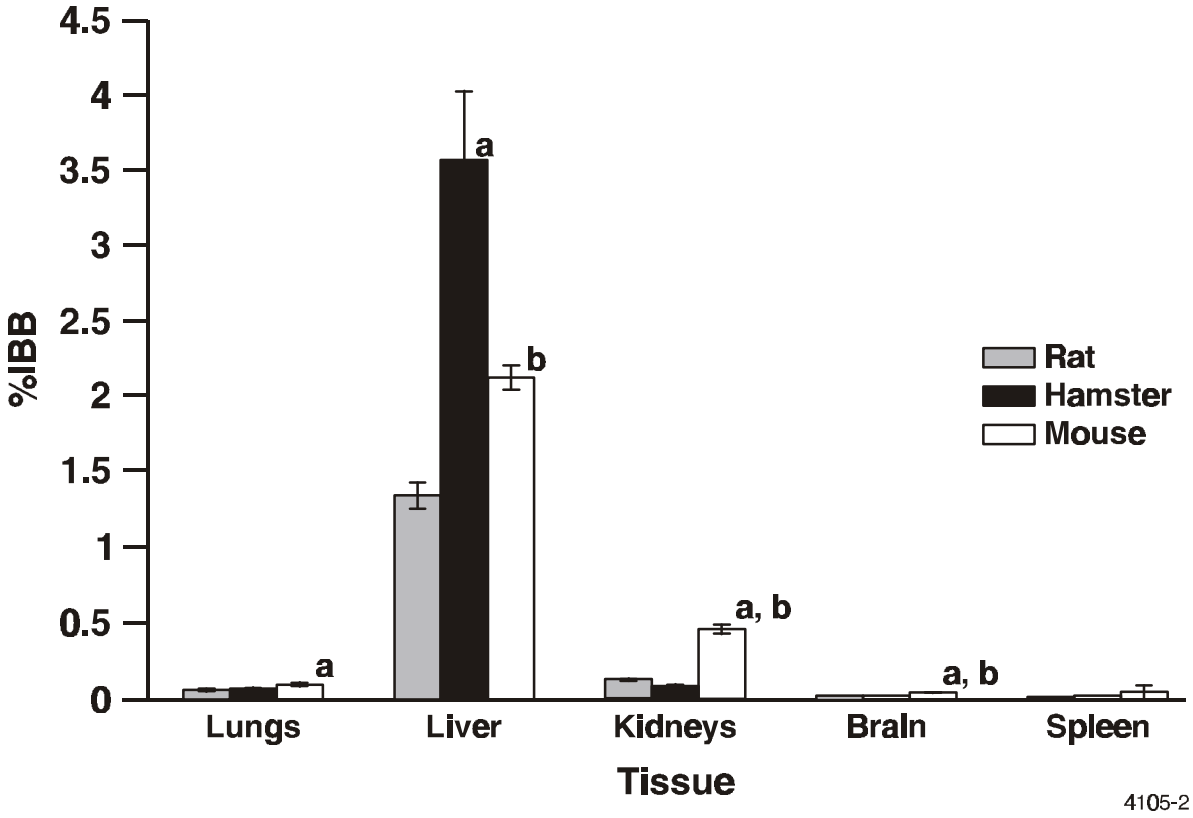
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
%IBB
a
b
a
a, b
a, b
Rat
Hamster
Mouse
Lungs
Liver
Kidneys
Braln
Spleen
Tissue
4105-2

Figure 11

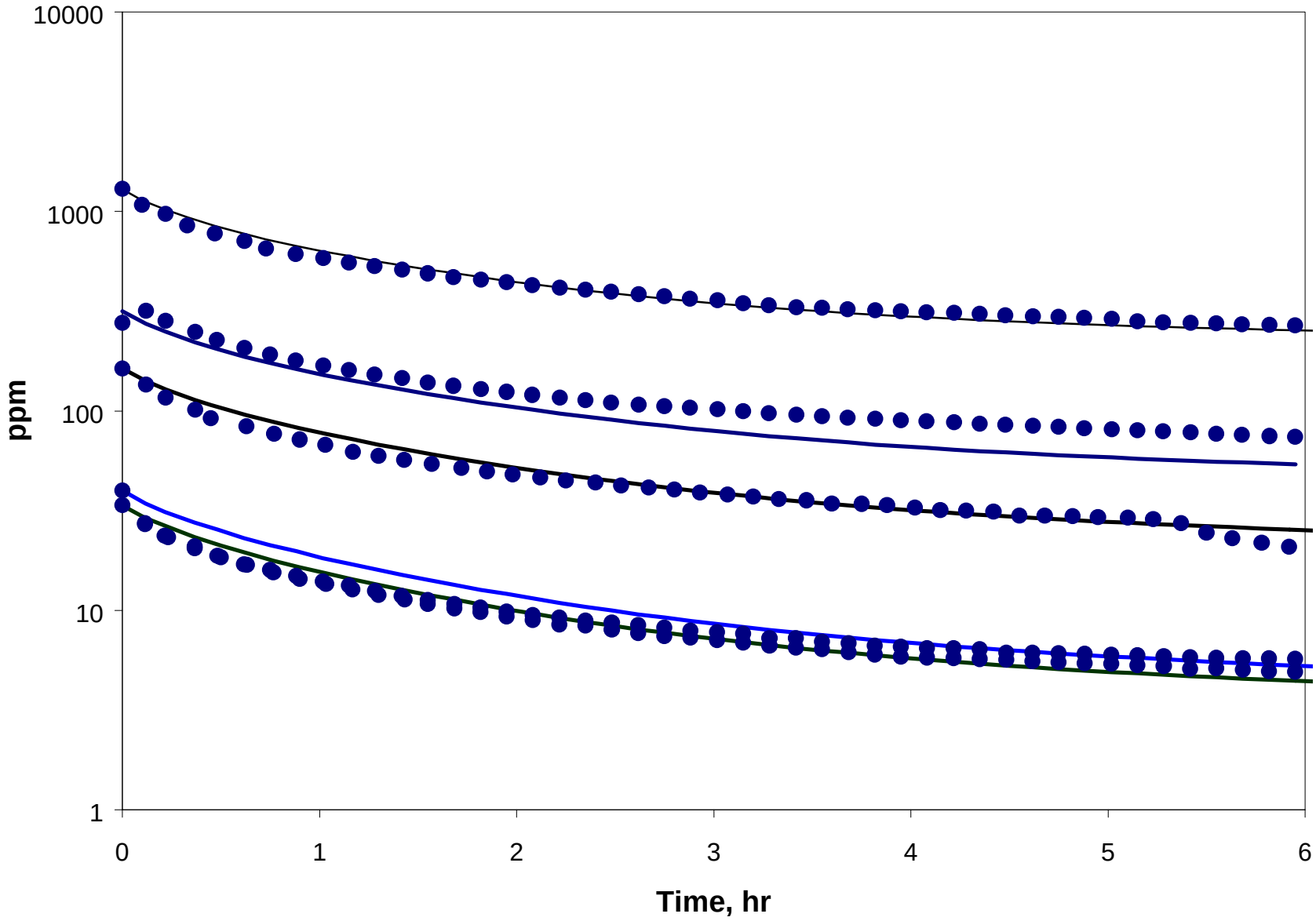

Figure 12

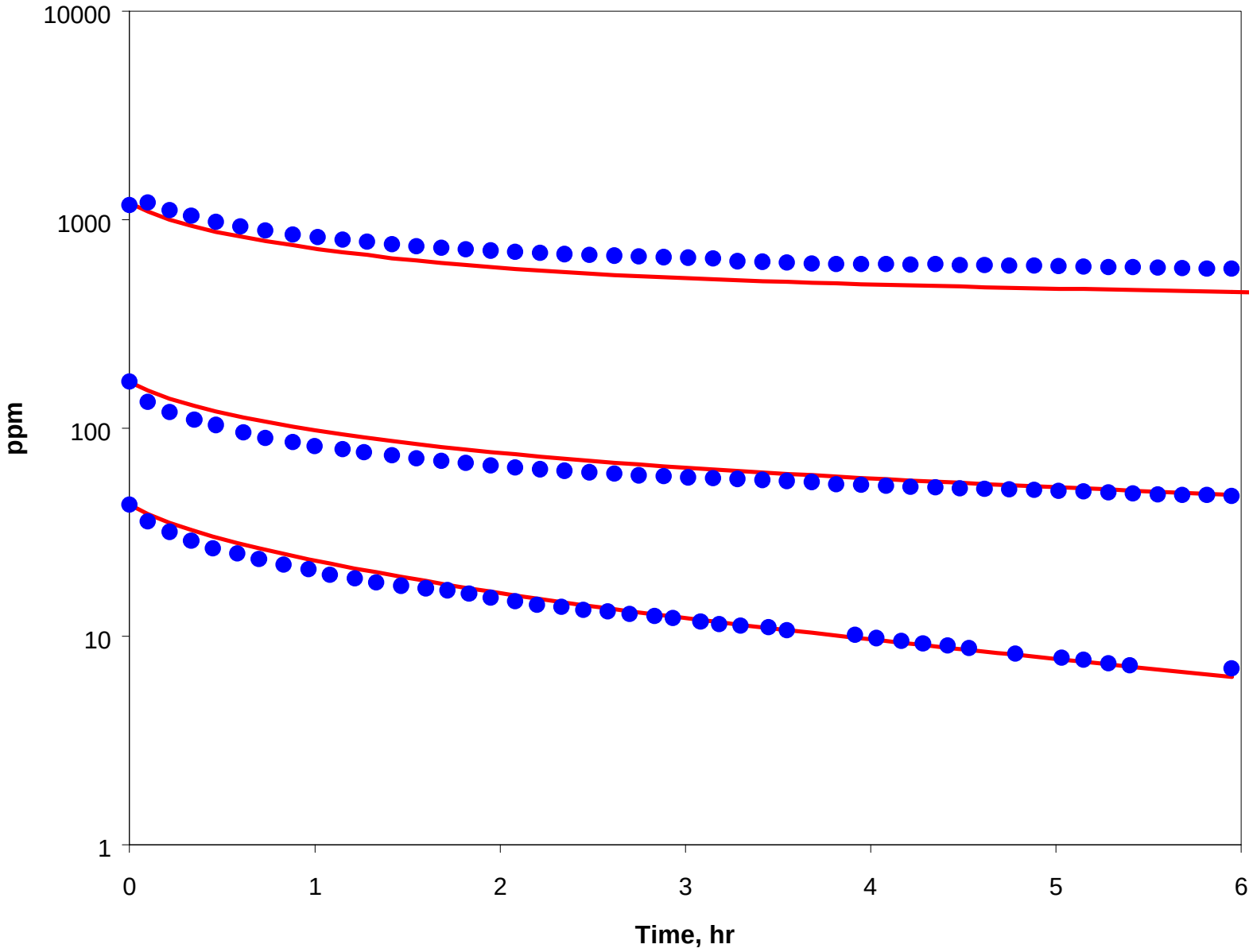

Figure 13

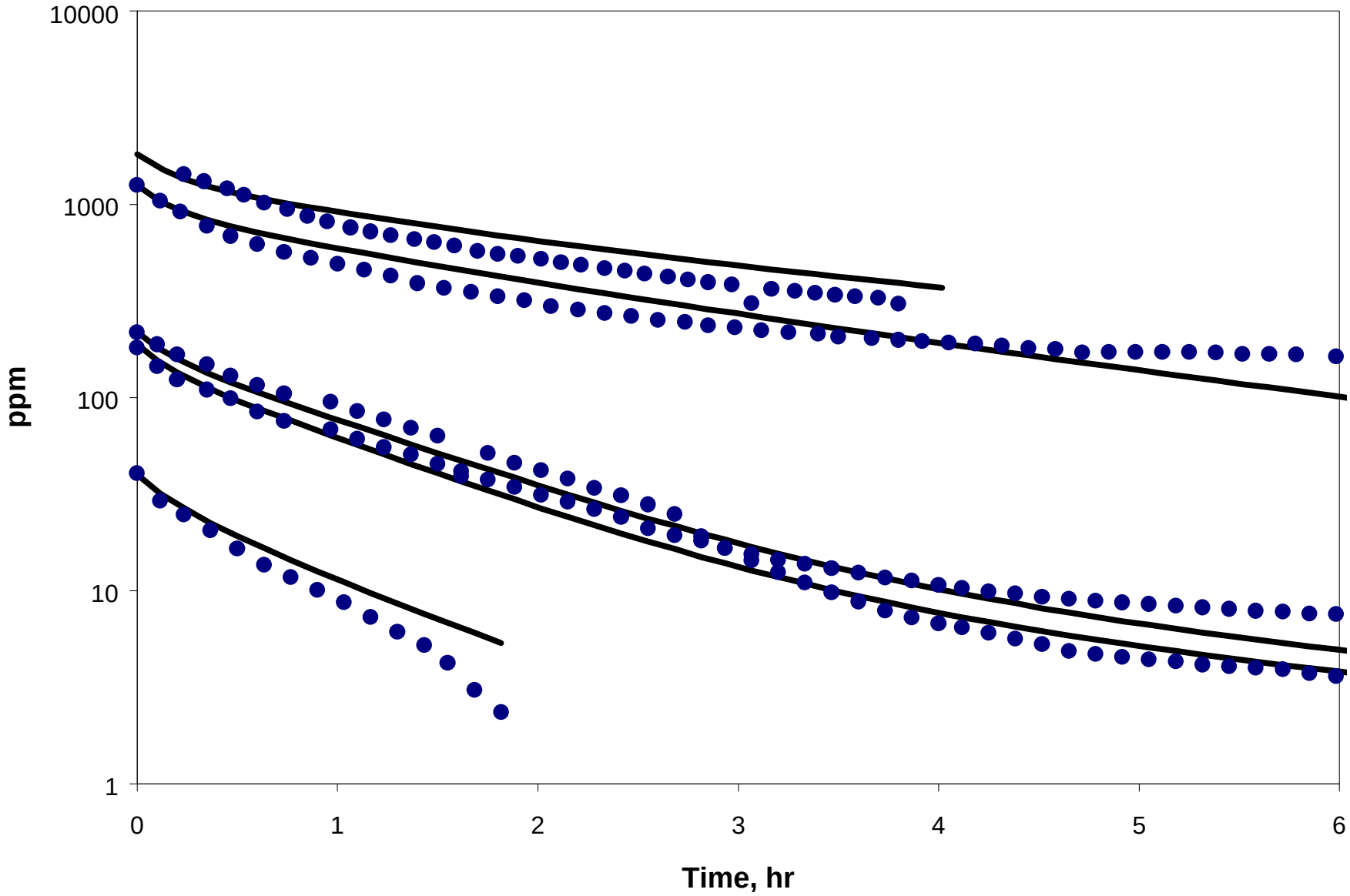

Figure 14

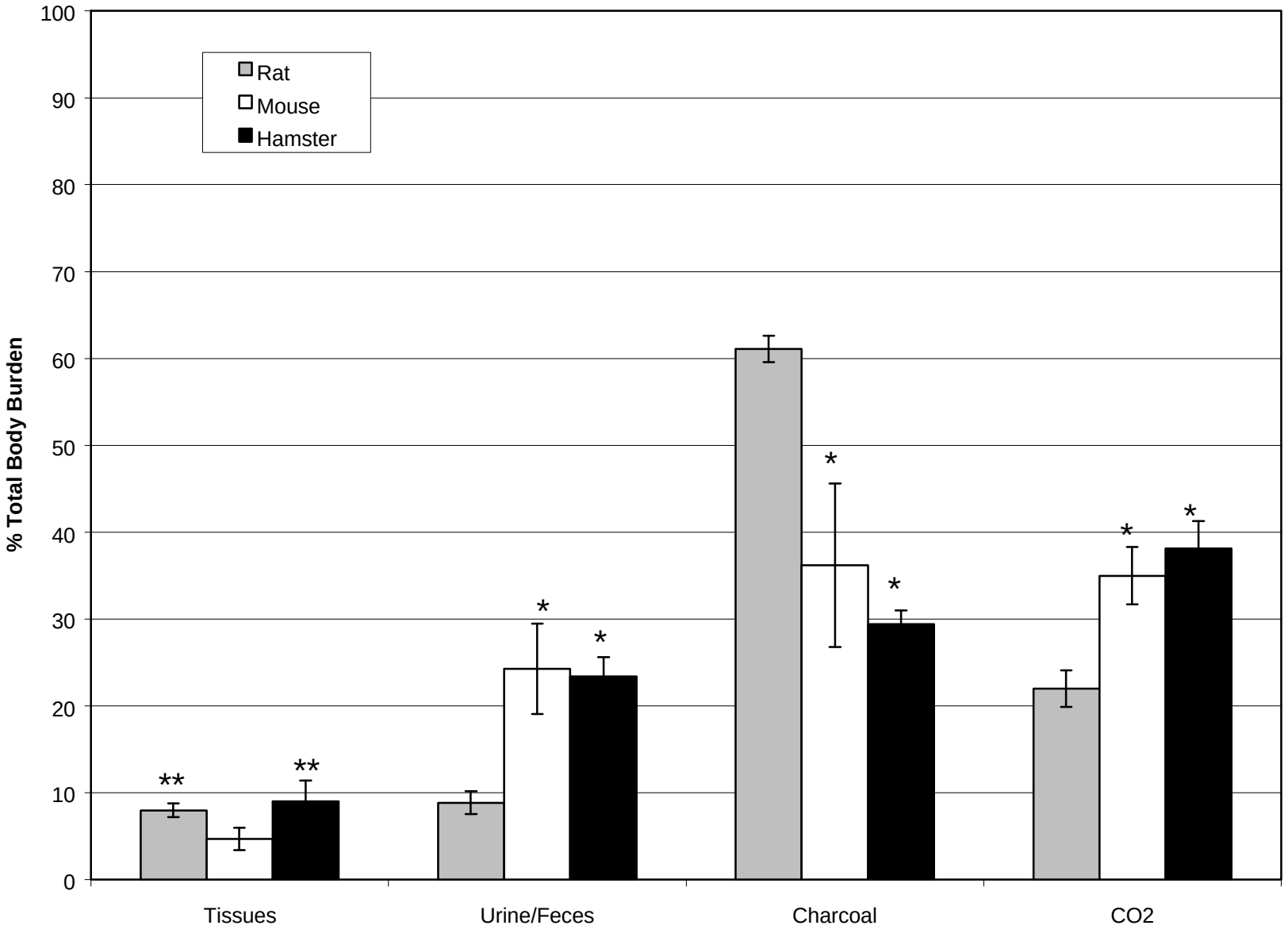
100
90
80
70
60
50
40
30
20
10
0
% Total Body Burden
Rat
Mouse
Hamster
**
**
*
*
*
*
*
*
Tissues
Urine/Feces
Charcoal
CO2

Figure 15

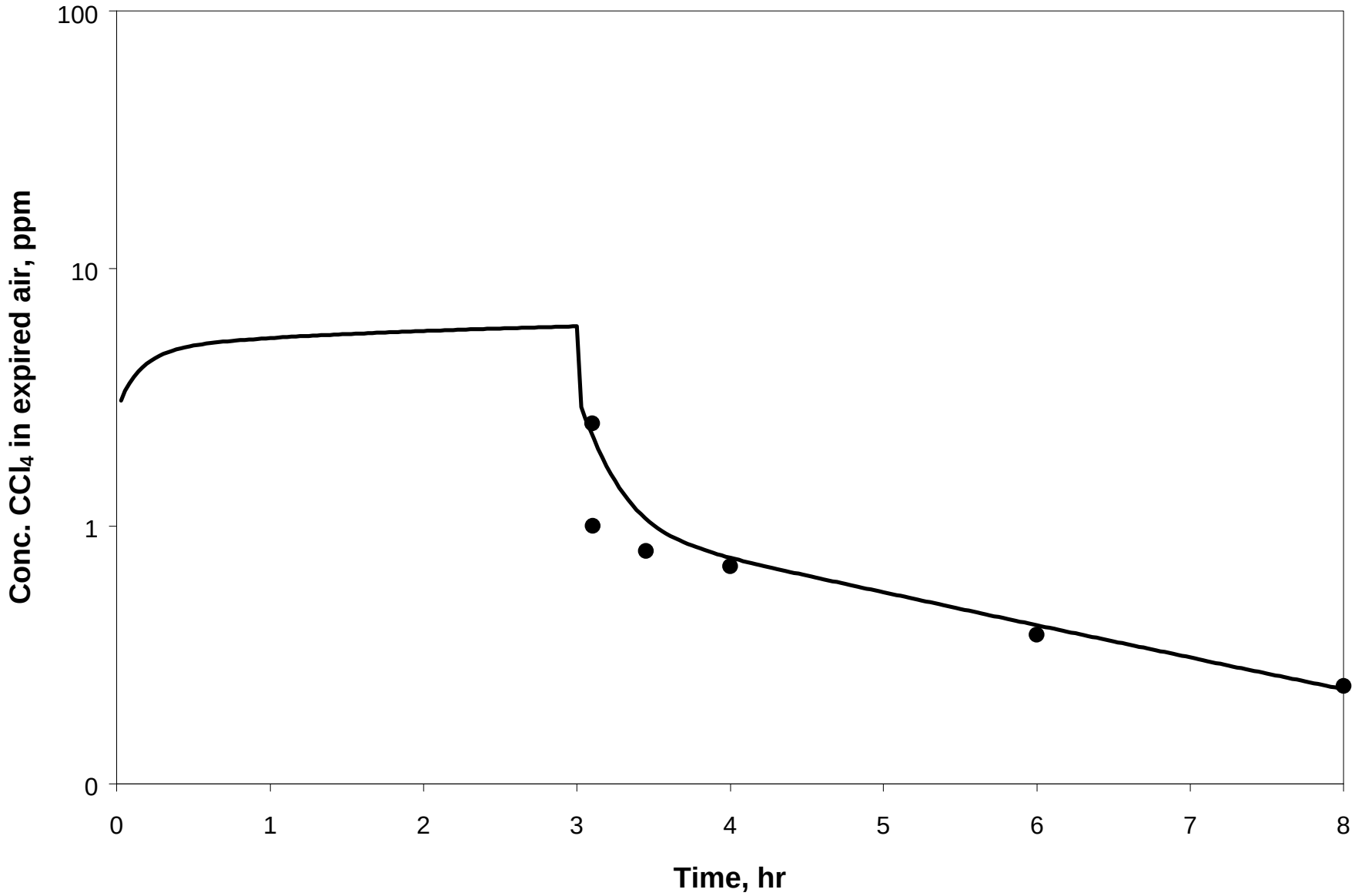

Figure 16

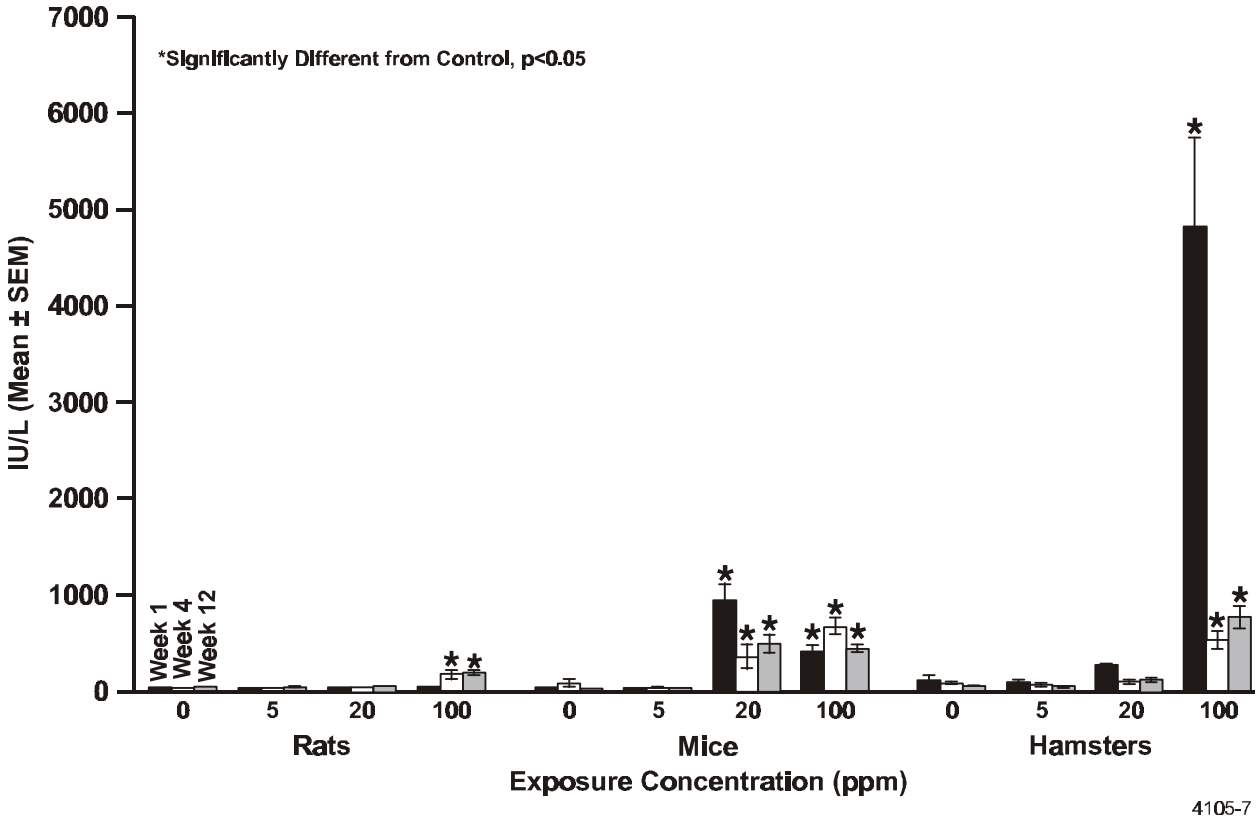
*Significantly Different from Control, p<0.05
IU/L (Mean ± SEM)
7000
6000
5000
4000
3000
2000
1000
0
Week 1
Week 4
Week 12
0
5
20
100
Rats
Mice
Hamsters
Exposure Concentration (ppm)
4105-7

Figure 17

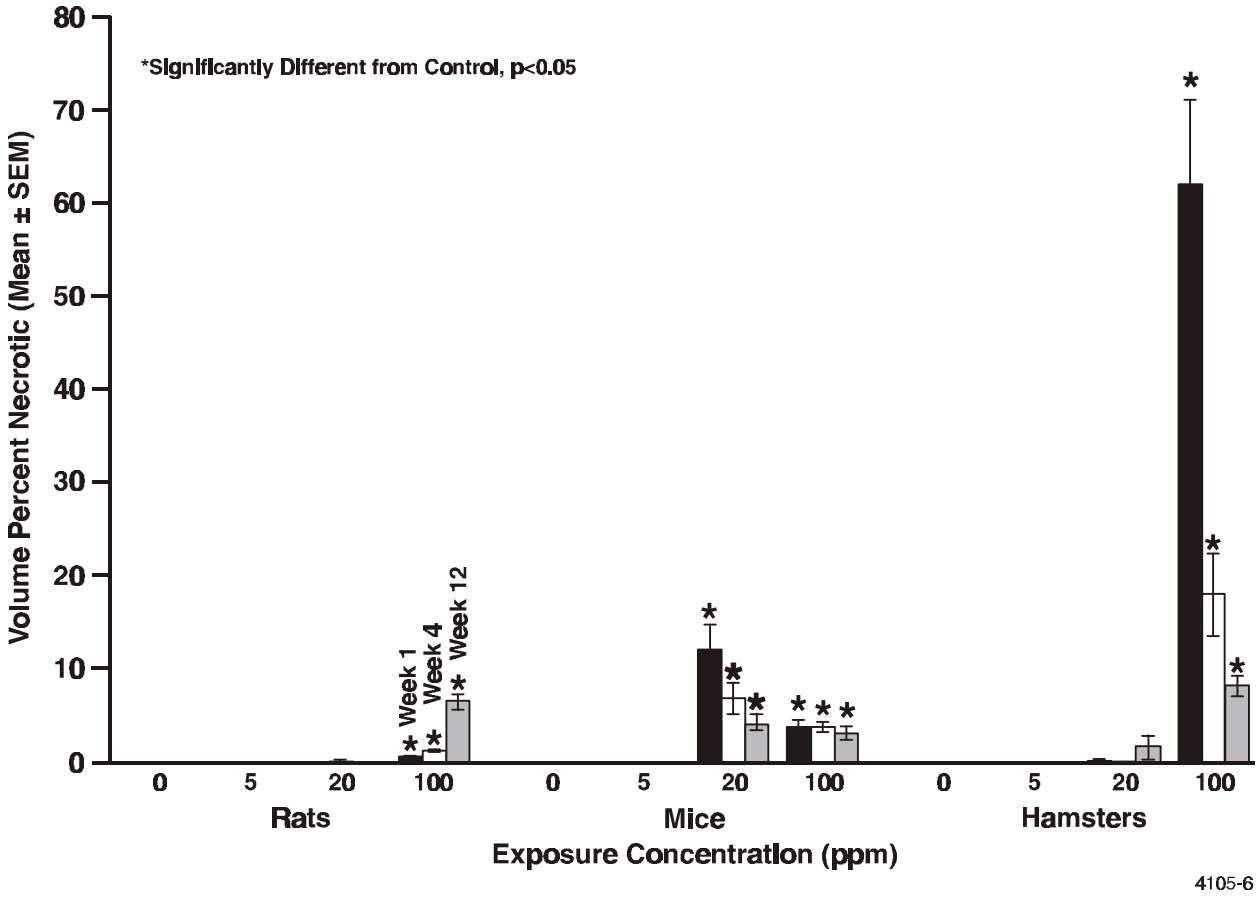
*Significantly Different from Control, p<0.05
Volume Percent Necrotic (Mean ± SEM)
80
70
60
50
40
30
20
10
0
Week 1
Week 4
Week 12
0
5
20
100
Rats
Mice
Hamsters
Exposure Concentration (ppm)
4105-6

Figure 18

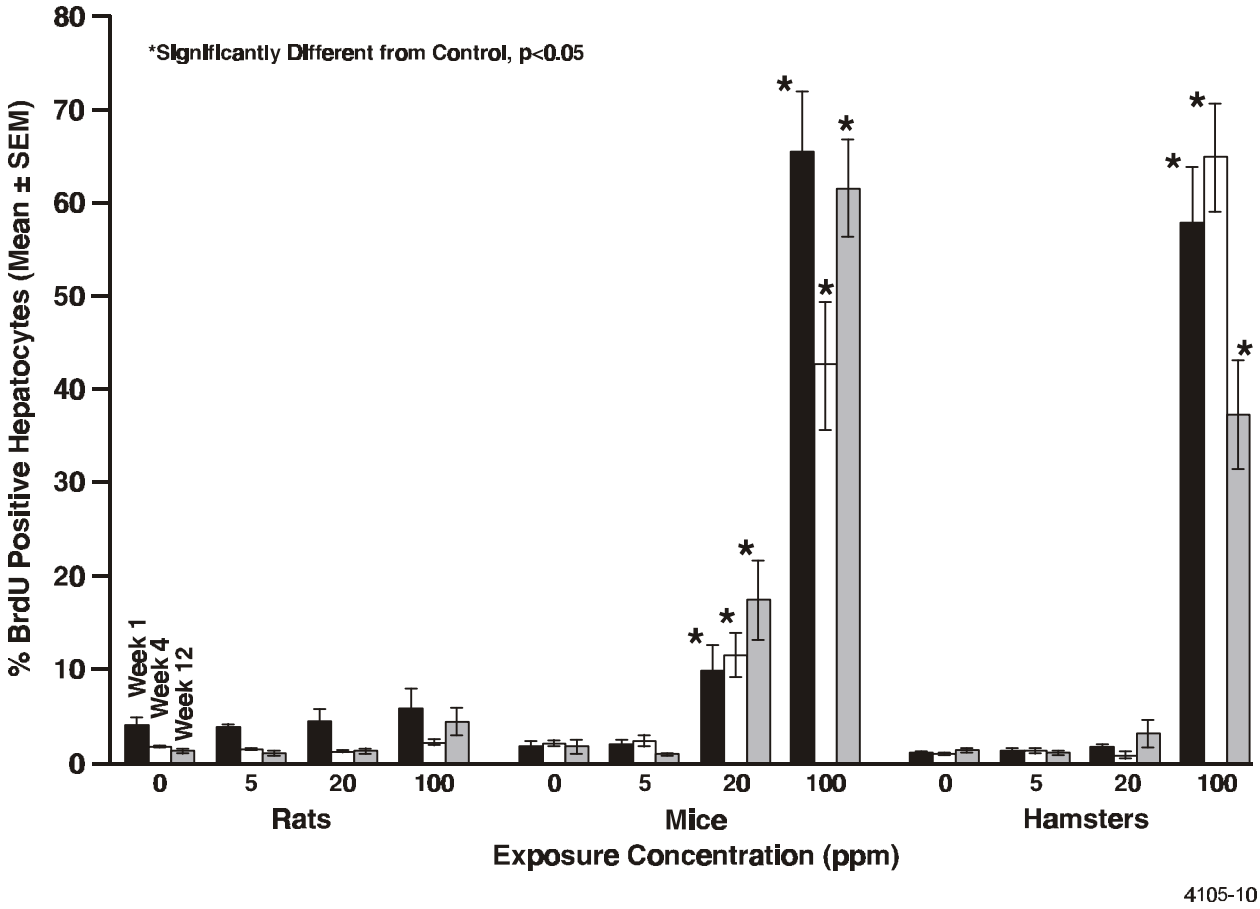
*Significantly Different from Control, p<0.05
% BrdU Positive Hepatocytes (Mean ± SEM)
80
70
60
50
40
30
20
10
0
Week 1
Week 4
Week 12
0
5
20
100
Rats
Mice
Hamsters
Exposure Concentration (ppm)
4105-10

Figure 19

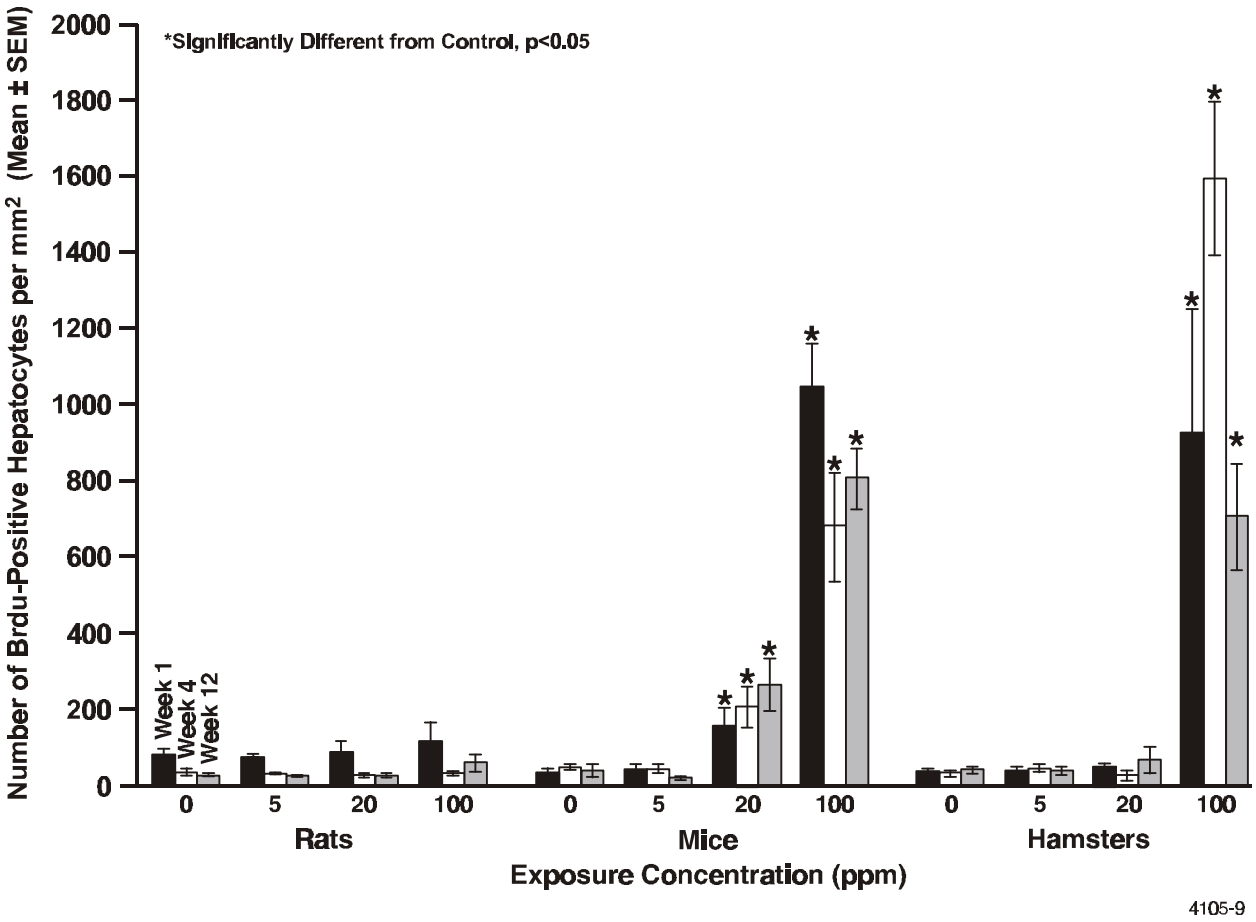
*Significantly Different from Control, p<0.05
Number of Brdu-Positive Hepatocytes per mm2 (Mean ± SEM)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Week 1
Week 4
Week 12
0
5
20
100
Rats
Mice
Hamsters
Exposure Concentration (ppm)
4105-9

Figure 20

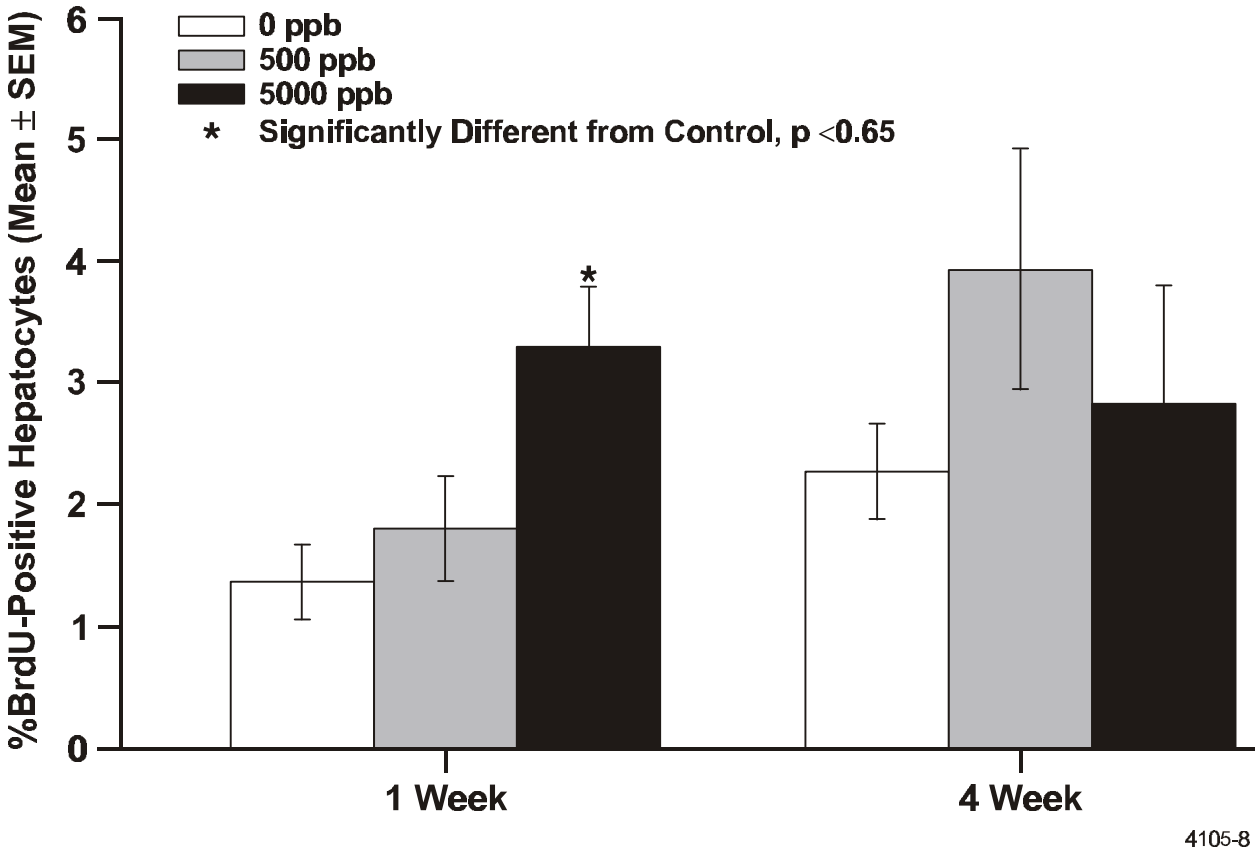
0 ppb
500 ppb
5000 ppb
* Significantly Different from Control, p <0.65
%BrdU-Positive Hepatocytes (Mean ± SEM)
6
5
4
3
2
1
0
*
1 Week
4 Week
4105-8

Figure 21